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THE NATURAL RADIOACTIVITY OF THE HUMAN BODY

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THE NATURAL RADIOACTIVITY OF THE HUMAN BODY

W. V. Mayneord

I. Introduction

It would be difficult at the present time to find a subject of greater public and scientific interest than the radioactivity of the human body. Our knowledge of the levels of radioactivity existing at the present time both in and around ourselves is very defective yet such knowledge is essential if we are to make sound judgements, both now and in the future, as to the possible significance of any rise of activity which may occur with the development of great public undertakings or private industries dependent upon radioactive materials in their basic processes. We have already with us the problems of "fallout" from the testing of atomic devices and such questions as those of public safety which arose following the accident to an atomic reactor at Windscale.

Many of these problems have often been treated as though radioactivity in the environment or in ourselves was a new phenomenon, though in reality similar activity has always been present in all living things, and evolution has occurred in the presence of levels of radioactivity at least comparable with, and sometimes much greater than, those arising from modern nuclear developments. We must recognize that the human body is naturally radioactive, an activity arising largely from the presence within it of very small quantities of members of the natural uranium, thorium and actinium series, together with the presence of a radioactive isotope of potassium ^{40}K and very small quantities of ^{14}C and ^{3}H. This activity, as we shall see below, is already incorporated into the embryo, built up in the foetus and continually renewed and dissipated in the metabolic processes of the adult organism. This dynamic concept of the activity is essential if we are to understand the levels of radioactivity attained in human tissues and the conditions determining them. These natural activities are usually often at a level much below those usually thought to be injurious to the human subject and though interest in these levels has been greatly stimulated by the present concern about problems of radiation hazards, yet the radioactivity of the human body has an intrinsic interest of its

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own as a branch of metabolic studies closely correlated with the dynamics of other trace-metallic elements. The radioactive elements may, in general, be studied at very much smaller absolute amounts (10^{-14} g may be measurable and identifiable) than analogous but non-radioactive materials.

In this paper we shall be concerned not only with the distribution and possible significance of radioactive trace elements in human tissues but also in questions of their origins, and the amounts actually ingested and excreted by humans in their normal or abnormal metabolism.

II. EXPERIMENTAL EQUIPMENT

FOR many years the main experimental techniques used in the determination of traces of radioactive materials as, for example, radium, in a wide range of soils, waters and many other natural materials, required the estimation of the quantity of radon evolved when the material was dissolved, fused or otherwise treated to drive off the gas. To effect this separation completely requires considerable care and, in any case, it cannot be safely assumed that the radon is in radioactive equilibrium with its parent, radium. If progress was to be made quickly, many thousands of estimations were required, and some new technique was essential. We have therefore made use of a simple procedure for estimating total α-activity, as described below.

The material whose activity is to be measured is prepared by ashing and/or grinding to a fine powder. A hollow translucent tray is formed by stretching Scotch tape over one surface of a Perspex ring whose internal diameter is about 5 or 10 cm and whose thickness is about 1 mm. The inside adhesive surface of the tray so formed is covered with a thin layer of finely powdered nickel-killed zinc cadmium sulphide which serves as the α-detecting phosphor. The tray is filled with the powdered sample and sealed by further layers of Scotch tape stretched across its upper surface. We have investigated carefully such factors as grain size of both phosphor and ash, or leakage of radon or thoron from the tray, and shown their effects to be normally negligible. The mass of phosphor used is normally about 7·5 mg/cm^2. The samples may be stored indefinitely and repeated measurements made at appropriate intervals. Some of our samples have been watched in this way for over 2 years. This has proved of very great importance in the identification of some of the materials as the specimens may either rise, fall, or stay constant in activity. If a change is observed, as occurs, for example, with many plant materials, the rate of change enables measurements of the half-life of a radioactive component to be made, and gives valuable clues as to its nature, as well as the amount which may have

been lost in the ashing process. In certain instances it is possible to investigate the biological raw material and to show that the estimates made from the use of ashed material are valid, but care is always necessary. Nevertheless, the concentration attained by ashing is very useful and, in such studies as soft human tissues, essential, if sufficient counts are to be obtained even in 24 hr counting time. The sealed sample is presented to the photocathode of a photomultiplier in a light-proof head unit. In many instances a sample of diameter 11·5 cm is used, with an EMI tube of approximately 12 cm photocathode diameter. Preliminary investigation established that the relation between counting rate and area of sample seen by the multiplier is linear. The α-scintillations produce in the multiplier pulses of the order of a fraction of a volt which are fed into a conventional ring-of-three amplifier of high stability. β- and γ-ray pulses are eliminated by biassing and the conditions carefully chosen to give a plateau of counting-rate.

The backgrounds observed by us are normally about 2 counts/hr over periods of the order of 100 hr. Further details may be found in previous publications (Turner, *et al.* 1958 a, b). It may easily be shown that the number of scintillations observed (N_0) over an area A per unit time is

$$N_0 = \frac{NRA}{4}$$

where N is the number of α-particles emitted per unit volume of active material per unit time. In precise determinations account must be taken of the variation of R, the range of the α-particles in the material, with density and atomic number of the sample. In careful absolute calibration experiments a precision of ± 3 per cent is attainable, an accuracy greater than usually required in these experiments on variable biological specimens.

During observations on materials containing thorium and its daughters, at low counting-rates of the order of 10–100 counts/hr, it was noted that numbers of closely spaced pairs of counts occurred although the chance of such pairs at these counting-rates is very low. The observations of double scintillations from the thorium series goes back as far as 1910, in which year they were reported by Geiger and Marsden. The disintegration scheme of the thorium series includes two consecutive α-transitions from thoron to ThA and from ThA to ThB (in modern nomenclature $^{220}\mathrm{Rn} \rightarrow {}^{216}\mathrm{Po} \rightarrow {}^{212}\mathrm{Pb}$). Since the half-life of ThA is only 0·16 sec the two α-particles occur as a pair with a mean spacing of about 0·2 sec. Careful consideration must be given to the geometry of emission of these two particles and the probability of chance pairs, but suffice it to say that the phenomenon enables the

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presence of the thorium series to be proved and, assuming conditions of equilibrium, to estimate the fraction of the activity arising from the uranium and thorium series, respectively. It is probable that an extension of this technique to the study of fast triplets may give information concerning the somewhat elusive actinium series.

In this way the absolute α-activity of most biological materials may be measured, the sensitivity being such that 1 count/hr above the background corresponds, in our equipment, to 4×10^{-14} c/g of sample. Soft human tissues may be measured, though the α-activity will often be only of the order of $3{\cdot}0 \times 10^{-13}$ c/g ash, or 5×10^{-15} c/g soft tissue.

While these techniques enable the total α-activity to be measured they do not of course identify the active material. In many experiments any naïve assumption of radioactive equilibrium among the members of a series was found to be fallacious and it became very important to improve the means of identification. Bearing in mind that the total amount of material may amount to only 10^{-14} c, it will be seen to be essential to attain very high efficiency of counting and, if possible, to identify every α-particle.

The most satisfactory general method of analysing α-activity is to measure the size of the individual pulses due to electrons which are produced when the α-particles are made to spend their full energy ionizing a free electron gas. Attention had hitherto been directed largely to materials having moderately high specific activity and the gridded parallel plate ionization chamber has been the instrument most commonly used. This type of chamber is not, however, suitable for the analysis of materials of very low activity, for which a large source area and a low background counting rate are essential. It is, however, possible to use a large cylindrical gridless chamber in which the sensitive volume is surrounded by material having very low α-emission and hence attain high sensitivity and at the same time low backgrounds. Details may be found in the literature (Mayneord and Hill, 1959; Mayneord, 1960).

The sources, which normally consist of a partially insoluble ash of uncertain chemical composition are prepared by grinding (by hand or in a ball mill) followed by spraying in the form of a suspension in water on to aluminized cellulose acetate film, previously wetted with dilute Teepol solution. A thin layer of sodium silicate solution is usually sprayed on top of the film so formed to act as adhesive. By this process it is possible to prepare sources which, under a microscope, appear to consist mainly of particles of about 1 μ diameter, spread with good uniformity. The acetate film has a very low α-emission of about three α-particles/hr per 1000 cm^2 compared to 100 for stainless steel, a material usually regarded as having low α-emission. The α-background

of such a counter filled with a mixture of 90 per cent argon and 10 per cent methane at atmospheric pressure consists very largely of radon coming from the steel walls of the enclosure. However, by continuously circulating the chamber gas over charcoal cooled in a solid carbon dioxide–alcohol bath, the radon background may be maintained in a large tank at some 70 counts/hr. The source may have an area of some 4000 cm^2.

Owing to the small particle size the initial absorption of the α-particles is small and by the use of suitable amplifiers coupled with a 100-channel pulse-height analyser the α-spectrum of many biological materials may be obtained. An example is given below (Fig. 4).

During the last 2 years these two techniques have enabled the writer and his colleagues to analyse thousands of specimens ranging from human tissues, both bone and soft tissues, human excreta (gaseous, liquid and solid), animal tissues, a wide range of plant material, hundreds of samples of soils, waters, gases, foods and many other environmental materials. In general, all contain detectable, and with few exceptions, easily measurable amounts of natural activity, chiefly of the uranium and thorium series.

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III. The Radioactivity of Human Tissues

It is very well known that one of the main radioactive constituents of the human body is radium and, in view of its chemical similarities to calcium, it is not surprising that a large fraction of the radium in the body is normally in the skeleton. Indeed, until recently it was thought that nearly all was in the bone.

There has been considerable controversy about the total quantity of radium in the normal human skeleton. In earlier work the value was quoted as high as 10^{-8} g of radium and therefore uncomfortably near the "maximum permissible" occupational level of 10^{-7} g, at which clinical symptoms might be expected. However, more recent investigations by Hursh and Gates (1950) and others have suggested a quantity of the order of 10^{-10} g of radium in the normal skeleton, the values ranging from 0·38 to $4{\cdot}3 \times 10^{-10}$ g ^{226}Ra. Walton *et al.* (1959) found the average for many localities in the world about 1×10^{-14} g Ra/g ash, but still variations by a factor of 10 within the specimens. We have recently been able to show the presence of the thorium series in addition to the radium. We shall not have time to discuss in detail the precise position of the radioactive material in the normal bone, but it is probable that the thorium series is taken up initially largely in the periosteum whereas the radium is distributed throughout the material of the bone more uniformly. The thorium series behaves more like plutonium. The microdistribution of the material in the normal case is

difficult to assess as the techniques of autoradiography are not sufficiently sensitive to be used, and any supposed analogy with distributions at much higher levels of activity attained by acute ingestion or feeding, in place of the slow accumulation and metabolism from normal foods and waters, may be misleading. To investigate these problems there is great need of a third technical development to carry the study of the spatial distribution of the ensuing disintegrations to much higher sensitivity than at present attainable by autoradiography. We will, therefore, be content to discuss here the absolute amounts involved. It has frequently been thought that as we grow older we take up radium, store it in the body and grow more radioactive. This, however, does not seem in general to be true, as indicated initially by Hursh and Gates and verified by us. Our results on seventy skeletons show no correlation between α-activity per gramme ash and the age of the subject. There are large variations from subject to subject, certainly over a range of fifteen to one, but no systematic change with age. This is also shown by a study of still-born foetal material. The twelve specimens examined by us indicate that even here the activity per gramme of bone ash is the same as in the adult (Fig. 1). The levels of experimental

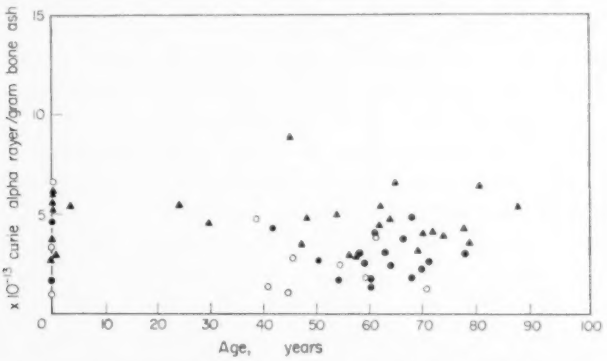

FIG. 1. Human bone ash: total α-activity. ▲ Cornwall; ○ London; ● Cumberland. (From *Nature, Lond.* **181**, 518, 1958.)

error are, of course, much less than the divergence between individuals. During the last few months my colleague, Miss Duggan, has studied the α-activities of a number of foetal samples of earlier age, the youngest being only 12 weeks from conception. These experiments are very difficult owing to the low calcium contents of the foetus but, again, the same result emerges, that the α-activity per gramme of ash is approximately constant. We shall see later that when we study soft tissues we again find approximately the same activity per gramme of ash and we seem to be in the presence of a kind of constant "contamination" of

radium in the building materials of the normal body. It will be noted that we have examined skeletons from three districts, namely Cornwall, London and Cumberland, and that the highest activities have been found in the Cornish specimens. At first one is tempted to correlate this variation with the known high uranium content of some soils in Cornwall, but we should be chary of such conclusions in view of the great variability of the radioactivity of foods and waters. Eating habits may be of great significance in determining skeletal activity. Through the kindness of the Department of National Health & Welfare of Canada we have also examined a series of ten specimens of Eskimo bones from Northern Canada. The particular interest of these specimens lay in the possibility that their high-protein diet might produce relatively low skeletal activity but, in fact, this does not appear to be the case, the values being rather higher than those derived from this country. It happens, too, that a number of the specimens from the London district were from the Royal Marsden Hospital and patients having advanced malignant disease. It could be that the well known decalcification which follows immobility, and is frequently associated with malignant disease, may result in the elimination of the radium from the skeleton to a greater extent than the calcium but this is not at all certain. The treatment of the malignant disease by chemotherapy may also involve the use of materials which might possibly act as chelating agents for some heavy metals such as plutonium, and this could result in increased elimination. Its significance with respect to radium would be worth investigation. It is not easy in such work to obtain adequate controls and in any attempt at using materials from fatal accidents other problems such as inadequate identification and history often arise.

The mean value of the α-activity of bone ash is in our experiments 0·4 $\mu\mu$ c/g of ash, so that the mean α-activity of the wet bone is very nearly $1{\cdot}0 \times 10^{-13}$ c/g. It is interesting to calculate that if the mass of the skeleton is 7×10^3 g, we have approximately 7×10^{-10} c of α-ray emitting material or, in other words, a disintegration rate of 26 α-particles/sec in the whole skeleton. A man of 70 years of age has thus suffered about $3{\cdot}3 \times 10^{10}$ α-disintegrations in his skeleton during his life. The production of a tumour by an α-particle is fortunately a rare event!

Measurements have also been made on the α-activity of human teeth. Again, it is found that there are variations from individual to individual certainly of the order of 20 to 1. The reason is again unknown. It is an interesting question, of some practical importance whether it might be possible to use teeth as a kind of biopsy material and so estimate the radioactivity of the skeleton. We cannot answer the question for the individual. The mean level of activity per gramme of

ash for our specimens of teeth is (within the individual variations) the same as that for bone ash, but we do not know whether or not this variation of activity from person to person is parallel in teeth and bone. It is, however, difficult to accept any picture of the erupted tooth in metabolic isolation.

It may be interesting to record that thanks to the British Museum we have made measurements on the rib of an Egyptian who died approximately 4000 years ago. The activity proves to be $3{\cdot}4 \times 10^{-13}$ c/g of dried bone. If due to the radium, the radium is 7×10^{-14} c/g dried bone—on the high side of normal. This is the only evidence we have that the natural α-activity of human bone 4000 years ago was much what it is now, certainly within the probable limits of variations between present day individuals.

It may also be worthy of mention that following studies of human skeletons it was natural to investigate the radioactivities of animals and, beginning with a series of cows, we have made preliminary observations on the bones of a few species (Mayneord, 1960). The very large differences observed are fascinating though, again, whether these differences are due to metabolic differences or eating habits or both we do not know. Man would appear to be a relatively inactive species.

Similarly, in studies of soft tissues we have observed some very high values, for example, in sheep, and these appear to arise from the high α-activities of grass, and in particular the presence in grass of relatively high levels of polonium.

IV. The Radioactivity of Soft Tissues

We now turn to the much more difficult problem of the measurement of soft tissues, since the activity per gramme of wet tissue is much lower than for bone.

We have obtained soft tissues at post mortem from seven subjects, six of whom were patients at the Royal Marsden Hospital. The procedure is, again, to ash the material and use the same counting method. The percentage ash for soft tissues is of the order of 1·5 per cent in place of 30 per cent for bone. Tables 1 and 2 show some of the activities recorded. In the normal subject the highest α-activity has been found in the thyroid and lungs with particularly high activity in hair. Most soft tissues in the human appear to have a total α-activity of approximately 0·4 $\mu\mu$ c/g ash. This would correspond to approximately 5×10^{-15} c/g of wet tissues or one disintegration per gramme of tissue in 1·5 hr. We see how rare an event an α-disintegration is even in 1 g of tissue. We will discuss this matter later in terms of the number of atoms of radioactive material per cell.

It is interesting to see the amounts of α-ray emitting material in the

TABLE 1

*Alpha Activity of Human Bones and Soft Tissues**

(Total α-activity c/g ash $\times 10^{-14}$)

Tissue Bone	*No. of Samples*	*Normal*	*No. of Samples*	*Radium Worker*	*Ratio of Activities*
Bone					
Femur, head	6	24·0	3	2100	87
shaft	8	25·6	—		
Humerus	15	32·0	5	2150	67
Tooth	2	17·6	1	1870	106
				Mean	87
Soft Tissues					
Lung	5	77·1	3	477	6·2
Lymph nodes, "clean"				152	
"dirty"				404	
Liver	7	33·6	4	134	4·0
Kidney	4	26·7	1	117	4·4
Thyroid	1	84	1	469	
Heart	5	28·3	3	103	3·6

* From *Clinical Radiology* **11**, 2 (1960).

TABLE 2

*Alpha Activity of Human Soft Tissues**

(Total α-activity c/g ash $\times 10^{-14}$)

Tissue	*Samples*	*Normal*	*No. of Samples*	*Radium Worker*	*Ratio of Activities*
Muscle	5	51·3	3	65·6	1·3
Spleen	6	40·2	3	30·6	0·7
Pancreas	2	18·1	1	31·2	1·7
Uterus	2	30·6	—	—	—
Bile	2	21·2	1	31·9	1·5
Brain	4	15·1	3	49·2	3·3
Testis	1	23·9	2	85·7	3·6
Prostate	1	30·5	2	123	4·0
Prostate	—	—	1	1400	Mean of soft tissues 3·1
Nodules	—	—	—	—	
Bladder	—	—	1	83·3	
Adrenal	—	—	1	92·2	
Hair	11	200			

* From *Clinical Neurology* **11**, 2 (1960).

various tissues of the body. Owing to its great mass (30,000 g) the muscle, having an activity of $0{\cdot}5\times10^{-12}$ c/g ash or 6×10^{-15} c/g of wet tissue, has a total content of approximately 18×10^{-11} c of α-rayer or about 3×10^{-11} c of radium–226 if in equilibrium. This is about 10 per cent of the value for the whole body. The blood with a mass of $5{\cdot}4\times10^{3}$ g contains about $1{\cdot}6\times10^{-11}$ c of α-rayer. Owing to its high specific content the lungs (10^{3} g) contain about $1{\cdot}3\times10^{-11}$ c of α-rayer, with other organs containing relatively little owing to their small mass. Lymphoid tissue has not yet been sufficiently investigated and much more work is required on the precise distribution of radioactive material in the reticuloendothelial system.

It will be noted that for comparison the tables contain fairly extensive results of our measurements of the tissues of a radium worker with an interesting history. He was an assistant in a famous laboratory at the beginning of the century and subsequently worked as a chemist on the production of radium. His main burden was probably acquired gradually some 40 years before his death at the age of 72. He developed chronic lymphatic leukaemia and died in 1958 of a coronary thrombosis.

Our colleagues at the Radiological Protection Service measured by external counting the radium burden in this patient while alive, giving a value of $0{\cdot}005\pm0{\cdot}002$ μc RaB+C, that is a total activity of the order of one hundred times that of normal. It will be seen how predominantly our results show the radioactivity concentrated in the skeleton. This raises at once an interesting point. There has been controversy about the percentage of radium located in the skeleton, most opinions having been derived from the study of radium luminizers or others having very large radium burdens. Muth *et al.* (1957) in the only soft tissue experiments of which we are aware found as much as one half of the radium of the normal body in the soft tissues, but this has never been verified. While we cannot agree with Muth and his co-workers that the activity of soft tissues per gramme of wet tissue is only two or three times below that of the bone, we do find with him a most striking difference between the patterns of concentration of radioactivity in the normal body and that of a body with a very large burden. The mean value of the ratio of the soft tissue activities of the radium worker to that of the normal is only 3·1, yet the skeleton contains roughly one hundred times the normal burden. Our experiments suggest that in the normal some 30 per cent of the α-activity is in the soft tissues while in the radium worker this may well be about 5 per cent. Of course, this ratio will probably be very different soon after heavy radium ingestion and before the material has had the opportunity to bury itself in an "unavailable" part of the calcium of the skeleton. We find, however, that study of the excretion by those with

long term high radium burdens fits with a relatively low soft-tissue content. Perhaps this is what a metabolic physiologist might have expected.

It is interesting to observe that if we record the activity per unit mass of ash rather than of wet tissue, the activity per gramme ash of soft tissues of the "normals" is, within the limits of the experiment, the same as that of bone. Perhaps we may interpret this result as implying an approximately uniform "contamination" of the mineral of the body by the radium, whether skeletal or extra-skeletal.

This result, of course, implies in the normal a ratio of about 20 between the amounts of radioactive material in the bone and soft tissues per unit mass of wet tissue. Tooth ash and bone ash show the same values within probable variations. Judged by the results per gramme of ash the highest soft tissue activities occur in the lung and thyroid. This is true of both the normals and also the radium worker. A particularly interesting result is that the spleen of the radium worker with lymphatic leukaemia is the only tissue having radioactivity below the normal values.

While ashing the prostate, my colleagues noticed that there were a number of probably calcareous nodules present which were, therefore, dissected out and measured separately. It will be seen that their activity is, indeed, very high and comparable with bone. It is difficult to resist the conclusion that this is calcium from the bone which has not returned to equilibrium with the soft tissues.

The lungs, of course, interest us very greatly as it may well be that some of the activity is derived from the atmosphere and may arise from particulate material caught in the lungs. We are starting to carry out more work on this subject.

A high value is obtained for human hair, the specimens now being derived, I hope, from really "normal" human subjects.

We have also been able to make observations on another set of tissues of great interest. A "radium luminizer" whose body burden was measured by the Radiological Protection Service and found to be $0{\cdot}008 \pm 0{\cdot}005$ μc Ra $(B + C)$ became pregnant, and at the normal birth of her child we were able to obtain a number of specimens. It seemed particularly important to study such organs as the placenta. This proved, indeed, to be one of the most active soft tissues we have yet encountered. The value of 220×10^{-14} c/g of wet ash tissue being six times the mean value of three normal placentas recently measured by us (36×10^{-14}). We do not, of course, know whether the placenta is a barrier against radium in the human as it is in many animals. It is interesting to record that the umbilical cord was less active by a factor of about 5, in agreement with the activities of many soft tissues, while

the amniotic fluid was so low in activity that we can only put it as less than 5×10^{-16} c/ml. Whether these results are to be regarded as proving little transference of radioactivity to the foetus we cannot be sure.

V. OBSERVATIONS ON NORMAL METABOLISM

IT seemed natural as the next step to try to ascertain what was the origin of the body's normal radioactivity and what fraction of the radioactivity ingested is retained. We have, therefore, made a number of observations very similar to the standard "calcium balance" investigations. The first was perhaps the most heroic. For a whole month we ashed and measured the activity of every food eaten by a boy 3 years old. No radioactivity was added and no change made in his normal diet. At the same time the activity of his faeces was investigated daily. Some of the results are shown in Fig. 2. Several important

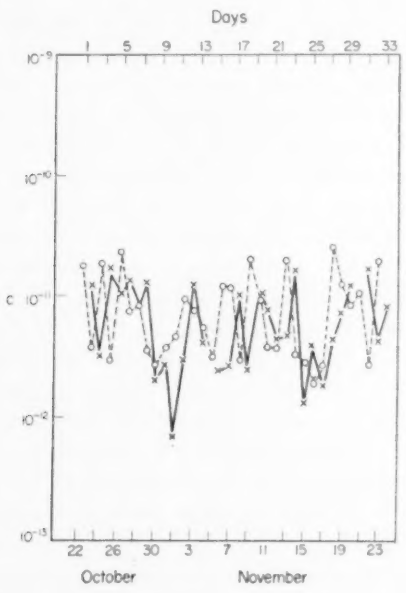

FIG. 2. Studies of α-activity of input and excretion of boy (From *Second United Nations Conference on the Peaceful Uses of Atomic Energy* 1958.)
Total α-activity (intake and faecal excretion).
Input TAA o——o——o.
Excreted TAA ×——×——×.
Excretion FEC 14 = 0.
Excretion FEC 30 = 0.

results emerge (Turner *et al.*, 1958; Mayneord *et al.*, 1958). It will be seen how greatly the amount of radoactivity taken in by the subject varied from day to day. By eating certain cereals for breakfast high values of intake were obtained, whereas a different choice of diet resulted in the intake being reduced by a factor of 10 or more. When the results of faecal activity were plotted against time it was interesting to see that the faecal activity follows that of the intake of the previous day. The results constitute one of the first hints we had that at least in some cases most of what goes in comes out again. When these results are plotted in an integrated form it is found that this boy appears to retain approximately 20 per cent of the natural α-radioactivity taken in. It is also possible to determine whether or not the thorium series (present to the same extent in both food and faeces) is treated by the body in the same way as the radium series. We have no evidence of any difference between them except that perhaps the thorium series is retained a little longer.

These investigations were repeated with young adult males, and the next figure (Fig. 3) shows the kind of results which have been observed. As we shall see, Brazil nuts are the food with the highest natural radioactivity so far encountered and, in fact, these Brazil nuts act as an admirable test meal for this kind of work. Specimens of urine and faeces for a number of days were carefully collected, and Fig. 3 shows how the activity varied in one instance. After the seventh day the subject ate about 120 g of Brazil nuts in a single meal and it will be observed that the activity of the faeces for the following two days is of the order of one hundred times its normal value. It falls again to normal in about 3 or 4 days. In this instance no radioactivity appeared to be finally retained, but we have repeated this experiment with varying results. In one instance, with another subject, 25 per cent seemed to be retained. It seems, therefore, that we still do not know what fraction of the radioactivity ingested is stored and one has the feeling that this may be a very complex matter depending upon the form of food and the calcium balance. We do not know if, for instance, radium phytate exists or what is the effect, if any, of vitamin D on radium absorption; what endocrine control is there of heavy element metabolism? Are they essentially dominated by carriers? When one considers the many hundreds of observations which have been carried out in order to answer similar questions about calcium, it is not perhaps surprising that we cannot yet be certain for radium.

These were, of course, observations on normal individuals. We may, however, raise one or two interesting points concerning the excreta of those having large radioactive burdens. In the United States a number of measurements of faecal excretions by radium

luminizers have been made and it has been shown that the faecal excretion gradually decreases with time after ingestion (Norris *et al.*, 1955). If, however, we look at the results and compare the absolute values with those we are now observing for normal persons, it will be found that after, say, 20 years the activity of faecal excretion

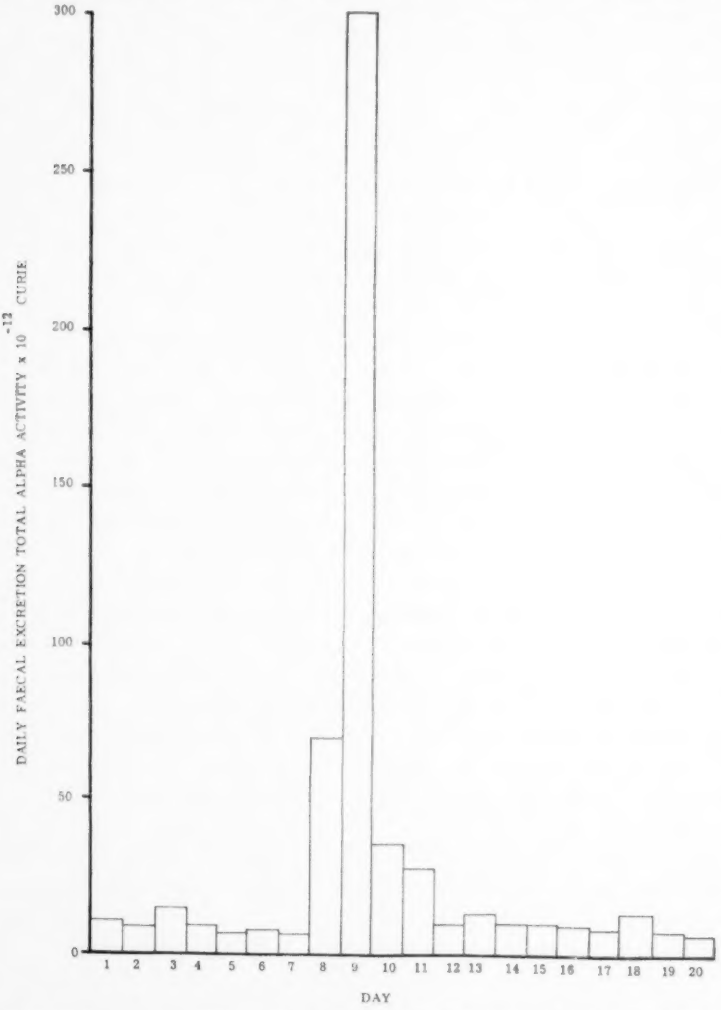

FIG. 3. Faecal excretion pattern before and after single intake of 120 g Brazil nuts. (From *Second United Nations Conference on the Peaceful Uses of Atomic Energy*, 1958.)
Total α-activity 380×10^{-12} c ingested immediately following faecal excretion seven.

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of persons having perhaps some hundreds of times the normal radium content of the body is reduced to approximately normal levels. The radioactivity of the faeces of those who have ingested large quantities of radium many years before could not compete with normal subjects a day or so after eating Brazil nuts. We should, therefore, be cautious in interpreting the results of faecal excretion in terms of radium body burden. In the case of the radium worker mentioned above we made measurements of faecal and urine activities several times before his death. The faeces were rather on the low side of normal ($2{\cdot}4 \times 10^{-12}$ c/day) but the urine slightly more active ($8{\cdot}5 \times 10^{-12}$ c/day). This is also true of the radium luminizer whose urine and faeces we have also measured; her faecal activity ($1{\cdot}8 \times 10^{-12}$ c/day) is, if anything, rather lower than normal, but the urine ($3{\cdot}5 \times 10^{-12}$ c/day) is rather on the high side of normal. We see that the correlation between radium content of the body and faecal or urinary excretion is a very complex matter deserving much more study.

As an example of an excretion of particular importance we have made measurements on both cow's and human milk. The activity of cow's milk was measured in some of our earliest experiments, and the mean value of nineteen specimens found to be 24×10^{-14} c/g wet ash. Our mean value for human milk derived from eight samples is 40×10^{-14} c/g ash. A single sample of the milk from the radium luminizer contained 240×10^{-14} c/g ash. It will be observed that the activity in normal human milk per gramme ash is in agreement with the mean of soft tissues, while the luminizer's milk is some six times higher.

We thought it would be interesting to find out whether the activity of human milk varied after a meal of Brazil nuts, and a nursing mother on a normal diet volunteered to furnish serial specimens of milk after feeding her child. In a second series of investigations on the same nursing mother serial specimens were obtained after the ingestion of Brazil nuts. Suffice it to say that we were not able to detect any rise of activity in the milk up to 72 hr after the ingestion.

VI. The Radioactivity of Foods

It has frequently been thought that drinking waters provide the main source of intake of radium for humans, and in some instances, there is evidence that this may be so (Marinelli, 1958), but during the last year or two my colleagues and I have measured the activities of about 200 types of food. The details have been published (Turner *et al.*, 1958c) and we include here merely a table (Table 3) which covers the wide range of activity observed. Brazil nuts are the most α-active food we have encountered. We have, indeed, very recently succeeded in obtaining the α-ray spectrum of this material, which shows (Fig. 4) the presence

of the radium and thorium series just as we had predicted from our observations of "fast pairs".

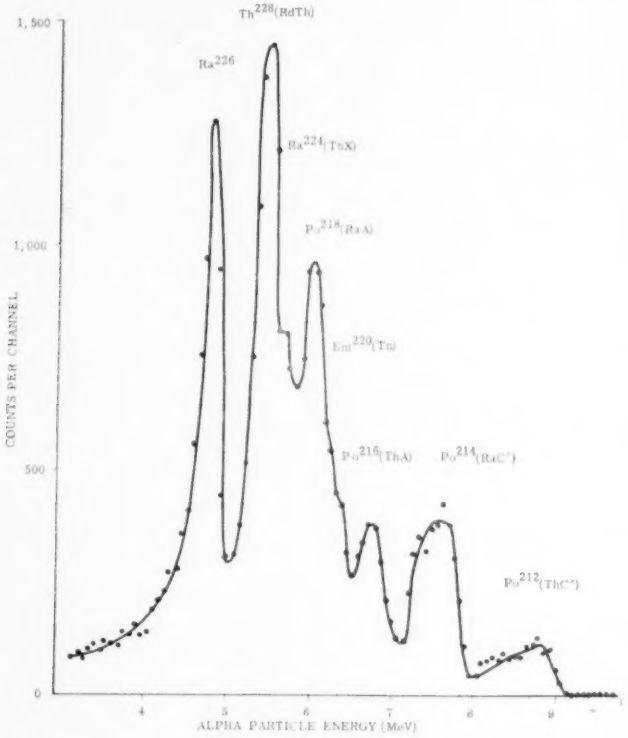

FIG. 4. α-Spectrum of Brazil nut ash. Counting time, 24 hr (From *Clin. Radiol.* **11**, 2, 1960).

Next in order of activity among the foods come certain breakfast cereals. Materials like teas are also high, though this is possibly in line with other observations that natural α-radioactivity is heavily concentrated in plants in rapidly metabolizing leaves. It may be a little comfort to add that only a small fraction of the activity is in fact taken up in the final infusion! It will be noted that liver and kidney are next in order of activity. Evidently in animals as well as humans these are sites of fairly high soft tissue values. On the whole the activities of milks, fishes, and proteins tend generally to be very low, with fruit and vegetables so low as to be barely measurable.

Generally a diet having high cereal content would be expected to be relatively radioactive, while one that is high in proteins will tend

TABLE 3

*Total α-Activity, Radium-226 Activity and Radiothorium-228 Activity in Foods (Arranged in Order of Decreasing Magnitude)**

Foodstuff	*No. of samples*	*Total α-activity/* 100 g × 10^{-12} c		^{226}Ra/ 100 g × 10^{-12} c	*Rad-*^{228}Th/ 100 g × 10^{-12} c	*Rad-*Th/Ra
		Max.	Min.	Max.	Max.	
Brazil nuts	4	1690	445	273	120	0·4
Buttered brazils	1	290	—	52	17	0·3
Brazil nut chocolate	1	133	—	24	7	0·3
Marzipan brazils	1	105	—	14	10	0·7
Cereal 1 (U.K.)	1	58	—	6·8	6·1	0·9
Cereal 2 (U.K.)	3	48	12	6·2	4·6	0·7
Tea (China)	1	46	—	5·1	5·1	1·0
Tea (Indian)	1	39	—	4·8	4·0	0·8
Tea (Paraguayan)	1	37	—	4·3	4·0	0·9
Tea (Brazilian)	1	34	—	4·0	3·6	0·9
Cockles	2	31	11	—	—	Ra and Th series
Cereal 3 (U.K.)	1	21	—	2·5	2·2	0·9
Flours	5	14·7	0·9	—	—	Variable
Peanuts	1	12·0	—	1·8	0·9	0·5
Peanut butter	1	12·0	—	0·9	1·7	2·0
Mussels	1	10·0	—	—	—	Ra and Th series
Chocolates (mixed)	4	8·0	1·4	—	—	Ra and Th series
Tapioca	1	7·6	—	—	—	Ra and Th series
Drinking chocolate	1	6·7	—	—	—	Ra and Th series
Molasses	1	5·4	—	—	—	Ra and Th series
Beverage 1	1	5·3	—	—	—	Ra and Th series
Cereal 4 (U.K.)	1	5·2	—	—	—	Ra and Th series
Cereal 5 (U.S.A.)	1	4·9	—	—	—	Ra and Th series
Curry powder	1	4·8	—	—	—	Ra and Th series
Cereal 6	1	4·6	—	—	—	Ra and Th series
Prawns (peeled)	1	4·4	—	—	—	—
Vichy water	1	4·0	—	—	—	No Th series
Cereal 7 (U.S.A.)	1	4·0	—	—	—	Ra and Th series
Cereal 8 (U.S.A.)	1	3·8	—	—	—	Ra and Th series
Sardines (tinned)	1	3·7	—	0·60	0·24	0·4
Cereal 9 (U.K.)	1	3·1	—	—	—	Ra and Th series
Breads (U.K.)	3	3·1	0·8	—	—	Ra and Th series
Cereal 10 (U.S.A.)	1	3·0	—	—	—	—
Cereal 11 (U.K.)	1	2·9	—	—	—	—
Walnuts	1	2·9	—	0·30	0·30	1·0
Soya	1	2·6	—	—	0·52	Th series only
Cereal 12 (U.K.)	1	2·4	—	—	—	Ra and Th series
Cereal 13 (U.K.)	1	2·4	—	—	0·50	Th series only
Haricot beans	1	2·3	—	—	—	—

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TABLE 3—(*contd.*)

Foodstuff	*No. of samples*	*Total α-activity/ 100 g × 10^{-12} c*		*^{226}Ra/ 100 g × 10^{-12} c*	*Rad-^{228}Th/ 100 g × 10^{-12} c*	*Rad*-Th/R
		Max.	Min.	Max.	Max.	
Biscuits	3	2·2	0·4	—	—	Variable
Baby rusks (U.K.)	1	2·0	—	—	—	Ra and Th series
Strawberry jam	1	1·9	—	0·24	0·19	0·8
Salami	1	1·9	—	0·20	0·20	1·0
Cereal 14 (U.K.)	1	1·9	—	—	—	Ra and Th series
Almonds	1	1·9	—	—	—	Ra and Th series
Calf liver	1	1·8	—	0·10	0·25	2·5
Cashew nuts	1	1·8	—	—	—	—
Salmon (tinned)	1	1·8	—	—	—	—
Sausages	2	1·6	1·1	0·20	0·17	0·9
Betel nuts	1	1·6	—	—	—	Ra and Th series
Cheese 1	1	1·5	—	—	—	Ra and Th series
Cereal 15 (U.K.)	1	1·4	—	—	—	—
Cereal 16 (U.S.A.)	1	1·3	—	—	—	Ra and Th series
Cheese 2	1	1·3	—	0·15	0·15	1·0
Margarine	1	1·3	—	—	—	Ra and Th series
Plaice	1	1·3	—	0·15	0·15	1·0
Cereal 17 (U.K.)	1	1·2	—	—	—	Ra and Th series
Hazel nuts	1	1·2	—	—	—	—
Evaporated milks (tinned)	2	1·2	0·7	—	—	Ra and Th series
Cereal 18 (U.K.)	1	1·1	—	—	—	Ra and Th series
Brazil coffee	1	1·1	—	—	—	Ra and Th series
Coconut	1	1·1	—	—	—	—
Fruit salad (tinned)	1	1·1	—	—	—	—
Cereal 19 (U.S.A.)	1	0·9	—	—	—	—
Processed cheese	1	0·9	—	—	—	Ra and Th series
Cheese 3	1	0·9	—	—	—	Ra and Th series
Parsnips	1	0·8	—	—	—	—
Pears (tinned)	1	0·7	—	0·11	0·06	0·5
Calf kidney	1	0·7	—	—	—	Ra and Th series
Bacon	1	0·7	—	—	0·14	Th series only
Ice Cream	1	0·7	—	—	—	Ra and Th series
Veal	1	0·7	—	0·09	0·06	0·6
Egg	1	0·7	—	0·20	—	Ra series only
Dried currants	1	0·7	—	—	—	Ra and Th series
Butter	1	0·6	—	—	—	—
Beef	1	0·5	—	—	—	Ra and Th series
Pistachio nuts	1	0·5	—	—	—	Ra and Th series
Cod	1	0·4	—	—	—	—
Tomato soup (tinned)	1	0·4	—	—	0·08	Th series only
Peaches (tinned)	1	0·4	—	—	—	Ra and Th series

TABLE 3—(*contd.*)

Foodstuff	*No. of samples*	*Total α-activity/* 100 g × 10^{-12} c		^{226}Ra/ 100 g × 10^{-12} c	*Rad.*-^{228}Th/ 100 g × 10^{-12} c	*Rad.*-Th/Ra
		Max.	Min.	Max.	Max.	
Pearl barley	1	0·4	—	—	—	—
Blackcurrant juice	1	0·3	—	—	—	—
Sago	1	0·3	—	—	—	Ra and Th series
Cow milks	20	0·3	0·08	—	—	Variable
Potatoes	1	0·2	—	—	—	—
Peas (tinned)	1	0·2	—	—	—	—
Lamb	1	0·1	—	—	—	Ra and Th series
Corned beef	1	<0·1				
Whale meat	1	<0·1				
Cream	1	<0·1				
Carrots	1	<0·1				
Sprouts	1	<0·1				
Mushrooms	1	<0·1				
Custard powders	2	<0·1				
Fruit jelly	1	<0·1				
Macaroni	1	<0·1				
Pineapple (tinned)	1	<0·1				
Banana	1	<0·1				
Spaghetti (tinned)	1	<0·1				
Apple	2	<0·1				
Tomato	1	<0·1				
Sugar	2	<0·1				
Rice	2	<0·1				
Cornflower	1	<0·1				
Beverage 2	1	<0·1				
Baked Beans (tinned)	1	<0·1				
Beverage 3	1	<0·1				
Beverage 4	1	<0·1				

* From *Health Physics* **1**, 268 (1958)

to be low. It must be emphasized, however, that although we have made thousands of measurements, the field is so enormous that in many instances we have only been able to measure one or two samples randomly chosen. It is not at all certain that one should speak generally of "the activity of bread or cereals", though the spread of levels of activity in a given category of foods is small compared to that in the whole range. However, when one looks at the results as a whole,

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one can see that an adequate Western diet will not, in general, contain less than about 5×10^{-12} c of α-activity/day. By ingesting some particular foods this amount could be raised by a factor of 1000. There is certainly no correlation of radioactivity of a food with its calcium content but there does seem to be a connexion with the phosphorus content (Turner *et al.*, 1958c), probably because the uptake by plants is correlated with their metabolic activities.

We begin to see one reason why there can be such large differences between the radioactivities of the skeletons of different people. The natural activities taken in from ordinary diet are, in general, much greater than the quantities of strontium-90 likely to be ingested.

VII. General Considerations

We have been carrying out these measurements for about 3 years and are naturally beginning to wonder whether any general picture is emerging. Personally, I have found considerable help from reading such books as those by Monier-Williams (1958), and Underwood (1956), for it becomes obvious that we are carrying out trace experiments with heavy metals, but with amounts perhaps a hundred million times smaller than detected in most work in this field.

The radioactivities of foods were the first to reveal the trace-element relationship. If one looks at the types of foods which have high α-activities they are also those which tend to have high contents of heavy metals. As a striking example, as long ago as 25 years Brazil nuts were known to contain no less than 0·2 to 0·3 per cent of barium, a result confirmed chemically in our own specimens by my colleague, Dr. Anderson. The nuts also contain high stable strontium but low calcium.

Some examples more nearly related to human tissues may be mentioned. The placenta is well-known to concentrate metals such as lead. As stated above, the placenta of the luminizer was relatively active. The thyroid was the highest of our "normal" soft tissues and was also outstanding in the tissues of the radium worker. It is known to contain relatively high concentrations of lead in the normal subject. Again, in the course of our observations on the tissues of the cow we measured the activity of the eye and found that the retina of the eye of the cow apparently has very high activity. This naturally interested us, and looking into the chemistry of the organ we found that the choroid of some animals, including the cow, has a high concentration of heavy elements such as barium. Beautiful work described by Pirie (1958) has shown this quite clearly. We have recently confirmed the high activity of the eyes of rabbits and sheep, and have no doubt that this concentration of radioactive material in the sclera, as well as in the iris,

is real. The human eye, not having tapital cells, has relatively less barium, and similarly we find rather smaller amounts of activity in the normal human eye though it is easily measurable (and greater than in most soft tissues) even in the very small masses of retina or iris available. We have also examined the eye of the radium worker, and again find an activity of the order of thirty times that of normal soft tissues. It is, moreover, well-known in beagle dogs having large radium burdens, that pathological changes occur in the tapetum, the choroid, and iris, perhaps associated with this high activity (Taylor *et al.*, 1958). There is a case for the careful investigation of the eyes of radium workers.

Following this apparent general tendency of the heavy metals and, therefore, α-activity to be associated with pigments we examined the specimens of human hair from eight individuals and find the whole material to have an activity of $3{\cdot}3 \times 10^{-14}$ c/g hair, about ten times the mean value for normal soft tissues. We are continuing these investigations to see if the material is deposited internally or externally and to determine whether hair is a useful biopsy material.

Recently, investigations have been carried out by Sowden and Stitch (1957) into the concentration of barium in human tissues and by Sowden and Pirie (1958) in the eye. It appears that the highest concentrations in human soft tissues occur in the thyroid and lung, the tissues in which we have found highest α-activity. It seems that a study of the metabolism of barium, known to be similar to that of calcium, might very well throw more light upon that of radium.

VIII. Absolute Levels of Activity

It is interesting finally to try to obtain a clearer picture of the absolute amounts of material detected in these measurements and their possible significance. We have shown that soft human tissues normally contain α-ray emitting materials at varying levels, but frequently of the order of 10^{-14} c of each emitter per gramme of soft tissues, this corresponding to some 5×10^{-10} c in the body as a whole. If 20 per cent of the radium is in the soft tissues we have in round numbers 10^{-10} c within the soft tissues. It is interesting to think of this in terms of individual cells. The "size of a cell" is, of course, extremely variable with type, stage of its life history and much else, but let us consider somewhat arbitrarily a cube of 5 μ linear dimension. Its mass will be approximately $1{\cdot}25 \times 10^{-10}$ g. We know that the α-activity due to radium is of the order of 10^{-14} c/g of wet tissue, so that if the material is "uniformly distributed" we should have 10^{-24} c of radium per cell. However, 1 g of radium-226 is approximately 1 c, and contains $2{\cdot}66 \times 10^{21}$ atoms. We therefore deduce that the domain of 5 μ linear dimensions contains 3×10^{-3} atoms, or that there is one atom of radium in about 300

"cells". If we take an alternative line of argument that the human body contains about 6×10^{13} cells we arrive at one atom of radium per 200 cells. The important point is clearly that these numbers agree in order of magnitude and it seems that in normal tissues we have a condition in which most of the cells will not contain even one atom of radium. For the short-lived members of the radioactive series the numbers are even more striking. Let us for the moment assume radioactive equilibrium in a series and, in accordance with the experimental results, assume 10^{-14} c of each member of the thorium series present per gramme in soft tissues. The numbers of atoms per curie of Th*A* (^{216}Po) is, owing to its short half-life of 0·16 sec, only $7{\cdot}5 \times 10^{-5}$ atoms per $\mu\mu$ c. It will be found that the chance of a "cell" containing an atom of Th*A* is very remote, only about five atoms being present in the whole body at a given time.

Again, returning to the radium-226 atoms, we remember that the half-life of such an atom is 1620 years, so that during the life of a cell in the human body (which may be very short, of the order of a few hours, and can hardly expect to last much more than 70 years) the chance of disintegration is small. A simple calculation shows that we may expect a disintegration in a given cell in about 800,000 years. It seems therefore that it is improbable that a cell in soft tissues will contain a radium atom and very improbable that this atom, if present, will behave other than as a stable atom. The Th*A* atoms will be present much more rarely but, if present, will almost certainly disintegrate.

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These numbers call to mind some of the basic questions. Can we expect in these circumstances the metabolism of radium at these levels to be dependent upon the chemical and physical properties of radium, or will they be dependent upon a carrier such as calcium present in overwhelmingly greater amounts? Certainly radium is a stable element in most cells. Again, in view of the long lives of some of the later members of the radium series, may not any damage by α-particle emission due to them be differently distributed from that due to the thorium series? If a thoron (^{220}Ra) atom disintegrates, its disintegration will be followed in seconds by two more α-particles and there will be a concentration of α-particles in a very small volume. In the corresponding case of radon (^{222}Rn) the times, or disintegrations, will be hours apart and there would seem ample time for movement of the atoms concerned. Very little consideration has been given to such problems, but they illustrate vividly the complexity of events in the sparse population of certain types of atoms as revealed by the application of sensitive methods of particle detection to biological materials.

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2

THE ENGINEERING APPROACH TO THE PROBLEM OF NEURAL ORGANIZATION

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THE ENGINEERING APPROACH TO THE PROBLEM OF NEURAL ORGANIZATION

A. M. Uttley

I. Introduction

In general terms the task of an engineer is to design a machine to have a given performance. The specification of behaviour comes first; for it the engineer has to deduce the necessary functions, and then to design equipment capable of performing them. The reverse process would arise if an engineer were presented with a novel machine and asked what it was for. This is a most unusual and uncertain procedure, particularly if the machine lies outside the experience of the engineer.

By analogy it is suggested that the engineer can best help, in the study of the nervous system, by studying known psychological facts of behaviour and attempting to devise mechanisms which will have such behaviour; his proposals may be regarded as physiological theories of neural mechanism. The engineer's theories will be of value only if they can be tested physiologically. Because of the language difficulty between psychology, engineering and physiology, it is very helpful if working models can be built; they form a check of the practicability of the engineer's theory and they form a kind of universal language between the three sciences.

In the present paper the behaviour considered is that of discrimination of sensory patterns and that of trial-and-error learning. A theory is developed of discrimination of binary signals by *classification*; physical classification systems are described; their lattice-like structure is compared with that of nerve tissue. A second theory is proposed of adaptation in a classification system which renders it capable of computing and storing conditional probabilities; it is shown that such a system is capable of trial-and-error learning. The necessary adaptation is described in terms of two proposed laws of variation in synaptic function; they are:

(1) After a neurone fires its axons become more powerful transmitters at synapses they form.

(2) After a neurone fires its dendrites become less sensitive receivers at synapses they form.

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The final section deals briefly with the much more complicated problem of discrimination of continuously variable signals by *measurement*. Corresponding to the principle of conditional probability is that of cross-correlation. It is pointed out that if the measure of some variable quantity is signalled as a variable impulse frequency, then nerve nets with the properties proposed above can compute the cross-correlation between pairs of variables.

In a nervous system energy of various forms falls on sensory receptors, where it is converted into a standard form, that of nerve impulses of varying frequencies which ascend a multitude of fibres; these signals are modified as a result of the stored after-effects of previous signals and eventually give rise to complex patterns of descending signals which control muscles and glands.

The first problem to be considered will be that of discriminating one set of ascending signals from another. The converse problem, which is similar in nature, is that of initiating one of a number of possible descending patterns of activity. The third and most difficult problem is to discover on what principles the stored after-effects of signals modify future ones.

Suppose that a stimulus causes three fibres to fire at three different frequencies, all of which may vary in a continuous manner. The total pattern of the signal may be said to vary in three dimensions. To store separately all possible signals and to distinguish them would require a three-dimensional storage system; a physical system to distinguish signal patterns in more than three fibres would appear to be unachievable. A further difficulty arises if there is any randomness in firing rates—and this is known to occur. It would not be possible at a single presentation to decide whether differences in firing rates were significant or merely due to chance variation. Consider the same problem at the stimulus level; a single coloured dot demands three variables for its subjective description so that the discrimination of one two-dot pattern from another would demand six-dimensional storage even if angular separation were ignored.

The perfect discrimination here envisaged does not seem possible—nor to be possessed—by living creatures; rather is it the province of scientific measurement. For these reasons the mathematical approach to the problem will be via a much simpler, and perhaps unrealistic situation. Suppose that, before discrimination is attempted, signals are transformed into a binary form so that only two states of a fibre are distinguished, either active or inactive. The problem now changes from one of measurement (of fibre frequency) to one of classification —of determining which set of all possible sets of fibres is active. If there are n fibres then there are 2^n possible such sets and a discrimination

system can be constructed which is simple in principle though astronomically large in size. We need not be perturbed at this because such a system would uniquely discriminate all possible signals at the first presentation, and we know that even man is incapable of this. A classification system whose size was limited to much less than the necessary 2^n units would have similarly limited powers of discrimination resembling those occurring in the innate behaviour of animals; we shall see later that only by introducing a principle of plasticity based on stored after-effects can there be discrimination which is adaptable to that set of frequently occurring patterns which is so much smaller than the almost infinite set of possible patterns.

As a first step, however, we must consider the principle of classification (Uttley, 1954) not only because it is simple but because it will be found to form a basis from which more subtle systems can be developed.

II. Classification

1. *Discrimination of incoming signals*

The principle of classification is defined in the following way. "A set (or class or aggregate) of objects is to be thought of not as a heap of things specified by enumerating its members one after another, but as something determined by a property, which can be used to test the claim of any object to be a member of the set" (Newman, 1951). It is important to note that there can be only one of two possible results of the above test; a property is either possessed or not possessed.

If, therefore, an external physical system causes activity in a set of channels, and if each channel can be in one of only two possible states —active and inactive—then that activity can be classified. Each channel can be regarded as indicating the result of a test on the external physical system and of distinguishing an elementary property of it; and so each channel defines a class of states of the external physical system. Two channels distinguish two classes; if both channels are active simultaneously that class is distinguished which is common to the two former classes; it is a sub-class of either. The larger the set of active channels (and of properties possessed) the smaller the class of activity being distinguished. A set of properties will be called *a pattern*.

A classification system with three input channels is sketched in Fig. 1. There are units connected to the inputs in all possible ways, one at a time, two at a time and three at a time; the function of each unit is to *indicate* if all the inputs connected to it are active simultaneously. Comparing the input signals to notes in music, each unit distinguishes a particular chord, though regardless of the presence of additional notes (properties); in a system with, say, seven inputs a to g, the (bcd) unit

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will indicate if there is activity, for example, in the set of inputs (*bcd*) (*bcdf*) or (*bcdeg*).

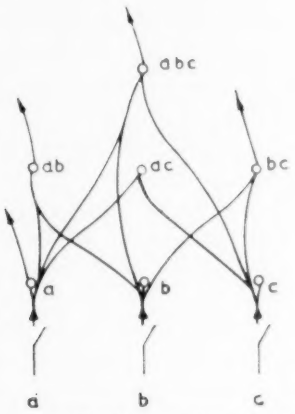

FIG. 1. A complete classification system for three binary inputs.

In line with the terminology of Set Theory, if one set of properties (pattern) contains another set it is called a superset of it. The corresponding indicating unit will be called a *superunit* of the unit which indicates the contained set.

If a two-dimensional array of light receptors were connected to a complete classification system there would be an indicating unit for every distinguishable shape; such a system could not, of course, generalize in respect of size or orientation.

In a system with complete classification, inputs would be connected to units in all possible ways and the connexions would show no particular structure; they could, indeed, arise from a principle of random growth. On the other hand, a classification system could be designed

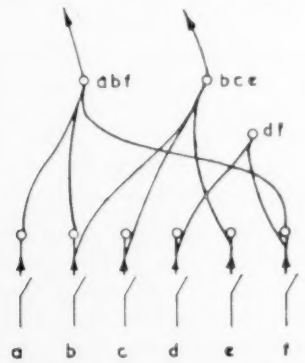

FIG. 2. An incomplete classification system for three binary inputs.

with special connexions to distinguish certain patterns only; Fig. 2 shows such a system, which distinguishes only the patterns (*abf*), (*df*) and (*bce*). It is suggested that it is such a special classification system which gives rise to Innate Releaser Mechanisms (Tinbergen, 1951).

So far only simultaneous patterns have been discussed; if spatiotemporal patterns are to be distinguished each input signal must pass through a series of delays after each of which a separate connexion is taken to the classification system; part of such a system is shown in Fig. 3. The (*ab'*) unit indicates if the connexions to it are active simultaneously; this will occur only if input *b* occurs before input *a*. Similarly, the (*a'b*) unit distinguishes the pattern *a* before *b*. Such a system can be extended to distinguish spatiotemporal patterns of any complexity; for example, receptors in a bird's retina could be connected through delays to a special system to distinguish the pattern "small image of hawk before large image" and "large image before small image". If the unit which distinguished the former pattern lay in the common path of a crouch reflex, this would be evoked only by objects moving towards the bird.

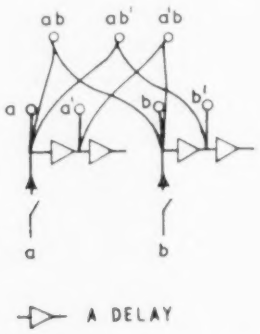

FIG. 3. A classification system for discriminating spatiotemporal patterns.

2. *Synthesis of outgoing patterns*

In a classification system a complex pattern of input signals activates a single key unit which corresponds to that pattern. Such a system can operate in the converse manner, as in Fig. 4 where the units form the input points; if any one unit is activated, a complex pattern of activity will occur at the lowest level of the system and this can form the output of the system. The classification principle is used here to synthesize patterns rather than to analyse them; by such means innate response patterns may be evoked.

3. *The indicating units*

A classification system can be constructed from units of a common design if each unit indicates when more than a critical number of the inputs to it are active; the critical number must be two or more. It is easiest to see this if the critical number is taken as two: the system of

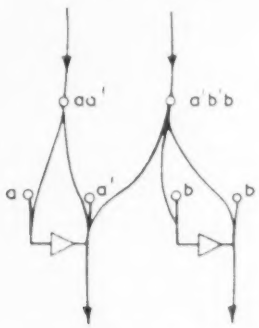

FIG. 4. A classification system for synthesizing spatiotemporal patterns.

Fig. 3 then takes the form of Fig. 5. There are two inputs to each unit; if they are active simultaneously an output of the unit is active and can serve as an input to further units. A classification system using units for which the critical number is ten takes the form of Fig. 6.

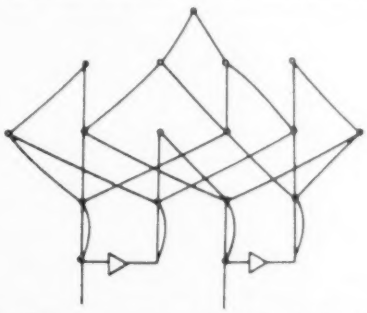

FIG. 5. A classification system constructed of identical units with a threshold of two input signals.

An essential feature of such systems is that each unit possesses the same fixed threshold; there is no storage of after-effects in the units.

A classification system can be complete, as in Fig. 7(a), and able to distinguish all possible patterns—this is a mathematical ideal; or it can be incomplete and designed to distinguish special patterns, as in Fig. 7(b); or it can be incomplete and yet have no special structure to enable it to distinguish special patterns—such an arrangement is shown in Fig. 7(c).

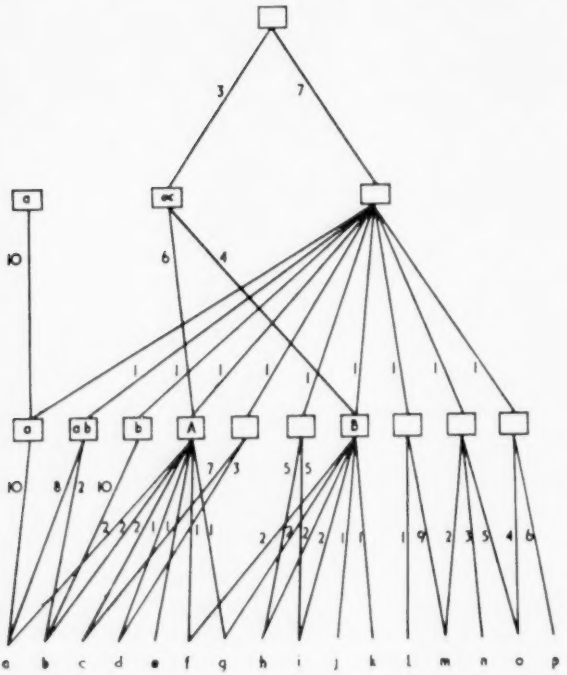

FIG. 6. A classification system constructed of identical units with a threshold of ten input signals.

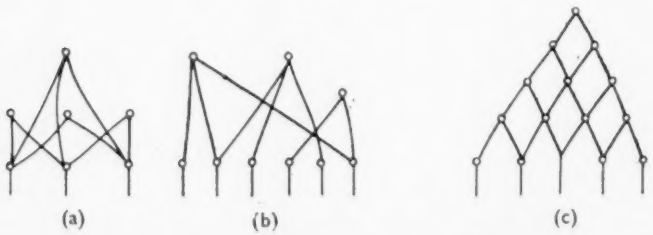

FIG. 7. (a) Complete classification of three inputs.
(b) Incomplete classification of specially chosen patterns.
(c) Incomplete classification arising because connexions are limited in length.

Under certain conditions a classification system could be formed in a population of randomly connected neurons. Consider a set of afferent fibres arborizing in a population of identical neurons each possessing a random dendritic system; the axonal systems of such neurons will not be considered at this stage. Suppose that synapses form by chance proximity and that a neuron fires if it receives two simultaneous

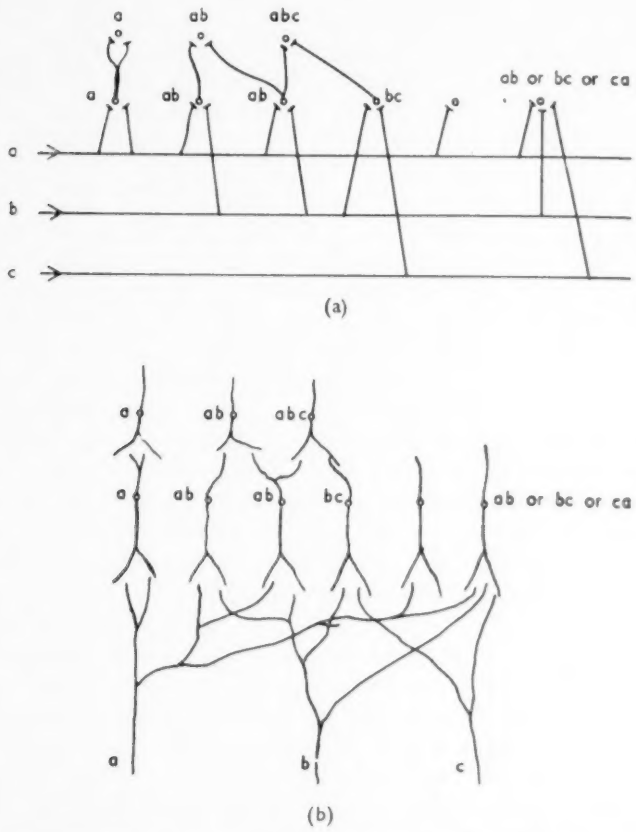

FIG. 8. Imperfections in classification due to random connexions; some units never indicate, some do so ambiguously. (a) Formal connexions to units which indicate if two inputs are active. (b) Neural realization of these connexions.

impulses. Such a system is shown diagrammatically in Fig. 8(a) and more realistically in Fig. 8(b); it possesses three features which do not occur in the formal classification system. Firstly there is more than one neuron with, for example, one connexion to an afferent fibre *a* and another to a fibre *b;* so, corresponding to the (*ab*) unit of the formal system, there is a diffuse cluster of (*ab*) neurons, and there is such a cluster for every unit of the system. The different clusters overlap but there is no confusion because each neuron is permanently labelled in terms of its connexions; it does not matter where a neuron is but what it is connected to. Because the units are duplicated such a neural

system could sustain much damage before it was unable to distinguish patterns. Secondly, there are neurons with less than two connexions; so they will never fire. In the limit, if fibres a and b are sufficiently far apart there will be no (ab) neurons, so classification will be incomplete; the actual number of neurons which distinguish the pattern (ab) will therefore depend upon the separation of the afferent fibres. Thirdly, if a neuron has connexions to more than two fibres it will indicate ambiguously, that is, when any two of the connexions are active.

At this stage it is important to define a *critically connected system*. Suppose that each neuron possesses a fixed threshold such that n synapses must fire simultaneously for it to be activated. Suppose also that there are only n synapses at each neuron. Then there will be no neurons which discriminate ambiguously and no neurons which never fire. Such a system will be said to be critically connected; this is, of course, a purely mathematical concept. Ambiguity of classification in an overcritically connected system is of special interest and it will be considered later in relation to variable thresholds.

If the neurons possess axonal systems these will make similar connective arrangements with the dendritic systems of further neurons. There are two consequences; firstly, there will be more duplication of indicating units; secondly, there will be units which distinguish more complex patterns.

The system can be extended to higher orders indefinitely, but it will be stable only under special circumstances. Consider only the (a) neurons of Fig. 8(b); the number which are indirectly connected to the (a) afferent fibre through one intermediate neuron might exceed the number of directly connected (a) neurons; similarly the second-order neurons might exceed the first-order neurons, and so on indefinitely; if the (a) fibre became active there would then be an uncontrollable increase in the number of active neurons. Each unit would then be an (a) unit OR a (b) unit OR . . . and there would be complete ambiguity of classification.

If, on the other hand, the magnification ratio were kept below unity so that the system was stable, then the actual number of neurons of a given order would decrease with the order. In consequence, as the complexity of a pattern increased the number of neurons which distinguish that pattern would decrease; but the nearer to the instability point that a system could be held, the greater the complexity of the patterns that it could distinguish.

The stability of a population of neurons has been studied by Beurle (1956) and Allanson (1956); this work is related to experiments on isolated cortex (e.g. Burns, 1951) and to measurements of fibre density (e.g. Sholl, 1953, and Eayrs, 1955).

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4. *Delays*

It has been pointed out that if there is a series of delays in each channel before classification then spatiotemporal patterns can be discriminated. This will also be true if the delays occur in the indicating units themselves.

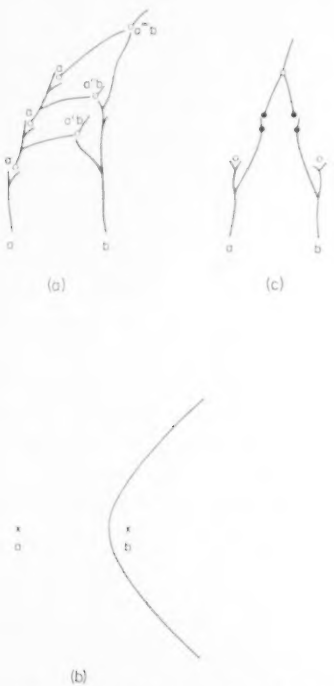

FIG. 9. (a) A suggested neural system for discriminating spatiotemporal patterns.

(b) General distribution in a neural sheet of neurons capable of discriminating a particular time interval between two impulses.

(c) Facilitation in a plastic classification system.

In Fig. 9(a) there is a chain of a neurons with increasing delays which combine with a single b axonal system to stimulate units $a'b$, $a''b$, $a'''b$, and so on. Such neurons will discriminate the pattern a before b, with increasing temporal separation between them. It follows that in a neural sheet stimulated at two points a and b with a given time interval there will be a tendency for the units which discriminate this pattern to lie along a particular hyperbola, as is shown in Fig. 9(b).

To summarize, a system of randomly connected neurons possessing

fixed thresholds can classify sets of binary signals in sets of fibres. If this overconnexion is carried too far any input will fire the whole system and ambiguity of classification will be complete, i.e. there will be no discrimination at all.

If there are delays in communication between neurons an incoming signal will cause the system to remain active for a time which will depend upon the degree of connectivity of the system. Such a system can classify spatiotemporal patterns.

III. Adaptation in Classification Systems

If there is a classification system with fixed connexions between units which have a fixed threshold, then the pattern of activity which each unit can discriminate is laid down and fixed. The number of spatiotemporal patterns which could occur in n sensory fibres with m discriminable time intervals is 2^{mn}, even if fibres were regarded as either active or inactive. If m is 10^6 and n is 10^2 the chance of one of these 2^{10^8} patterns being discriminated in a population of, say, 10^{10} units is negligible. One is led to wonder whether a system could operate on the principle of holding available a set of unconnected units and, by some plastic process, connecting them up for those patterns which do in fact occur frequently in a particular environment. This gradual increase in connectivity would demand the often-postulated principle of facilitation whereby use increases conductance; but it can be shown to be quite illogical.

Consider the simple situation of two channels a and b with an indicating unit for each and a third unit available for connexion if, in fact, a and b do ever occur together. The arrangement is shown symbolically in Fig. 9(c). The requirement now is to close the switches which connect the ab unit if ab often occurs. Clearly an ab unit is required to detect whether ab does occur. The proposal results, not in economy, but in a duplication of units. The same basic illogicality shows up in the facilitation theory. If, initially, the conjunction ab does not occur and no impulses travel from, say, the a unit to the ab unit, then the principle that the passage of impulses facilitates conduction between units can never begin to operate.

However, these illogicalities disappear if the converse principle is used. Suppose that units are initially overcritically connected, rather than undercritically, so that there is great ambiguity of discrimination. The ambiguity will be reduced if, by use, connexions become less effective; this demands adaptation, a widely occurring physiological phenomenon. As the connectivity is reduced it will tend to become critical so that unambiguous classification is achieved.

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Whether connexions are to be made or broken, some controlling principle is demanded which depends on the past history of incoming signals. That of conditional probability will now be considered; it can apply only if signals are binary but it can be employed either to facilitate or adapt connexions.

1. *The conditional probability hypothesis*

Suppose that the past history of three inputs a, b and c is as shown below.

a	1111100
b	0010111
c	0010011

From this data it can be said that when a occurs there is a probability $2/5$ of b occurring with it. Formally, the conditional probability of b on a, written $p(b/a)$ is $2/5$. Conversely, $p(a/b)=2/4$. Further, in the condition of a and c occurring together the conditional probability of b, $p(b/ac)$, is unity since, on the only occasion when a and c occurred, b also occurred. If the principle of induction, inferring the present or future from the past, is invoked, a system can be devised which will make predictions and act on them.

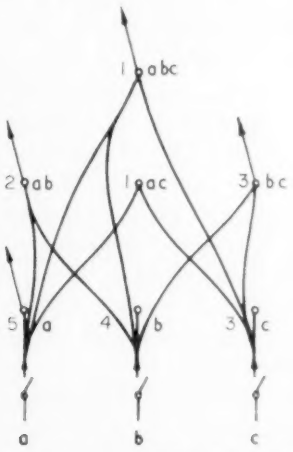

FIG. 10. The state of units in a conditional probability system after a particular set of signals (see text).

To calculate conditional probabilities one must count, in the above example, not only the number of times a, b and c have occurred—in this case 5, 4 and 3, but also the number of times that the coincidences ab, bc, ca and abc have occurred. It will be seen that a classification system contains all the necessary indicating units; the new feature

needed is that they shall count. For the above example, the units of the system shown below will contain the numbers indicated.

The system must operate in the following manner. If *a* occurs the *a* unit will indicate; when it does so it must cause the *ab* unit, to which it is connected, to emit 2/5, the ratio of the contents of the two units; similarly for the *ac* units. If, however, *a* and *c* occur, the *ac* unit will indicate and must cause the *abc* unit to emit the ratio of their contents —in this case unity.

Nothing has yet been said about the scale of counting, which need not be linear. An approximately logarithmic scale has two advantages in a physical system; it allows for a greater range between the maximum and minimum counts which can be stored; also, the determination of conditional probability becomes one of subtraction rather than division —a much simpler process in physical systems.

To summarize, a physical Conditional Probability System must operate in the following way. If a particular pattern of input activity causes a certain unit to indicate, then this unit must cause any *superunit* of it to emit, on some scale, the ratio of the count in the *superunit* to that in the indicating unit. This effect will be called *supercontrol*. It follows that units may become activated in two different ways, by actual occurrence of patterns of signals—as in a classification system— or by inference if they are highly probable.

A particular form of Conditional Probability Computer has been built which closely imitates a large number of forms of animal behaviour (Uttley, 1956). The scale upon which probability is scaled is arbitrarily chosen to be positive so that high probabilities are represented by large signals; it follows that when *a* occurs, a large signal is emitted from the *ab* unit if $p(b/a)$ is high. Since this signal represents the present probability of *b*, it should be transferred from the *ab* unit to the *b* unit. Such a flow of signals, from superunits to subunits will be called *subcontrol*. The computer shown in Plate 1 has five input channels *j*, *k*, *l*, *m* and *n*, connected to thirty-one indicating units in all possible ways on the lines of Fig. 1. Each unit stores, on an electrical condenser, a voltage roughly proportional to the logarithm of number of times that the unit has indicated. There is a resistive leak across the condenser which causes the effect of past counts to leak slowly away. If any input, say *jk*, occurs, the voltage stored in the *jk* unit is subtracted from all the superunits of *jk;* this is supercontrol. It follows that the *jklm* unit, say, will now contain a voltage which is the logarithm of the number of times *j*, *k*, *l* and *m* have occurred together *minus* the logarithm of the number of times *j* and *k* have occurred together—in other words, log $p(lm/jk)$. By subcontrol, this quantity is transferred to the *lm* unit which now contains a measure of the likelihood that *lm* can be inferred from *jk*.

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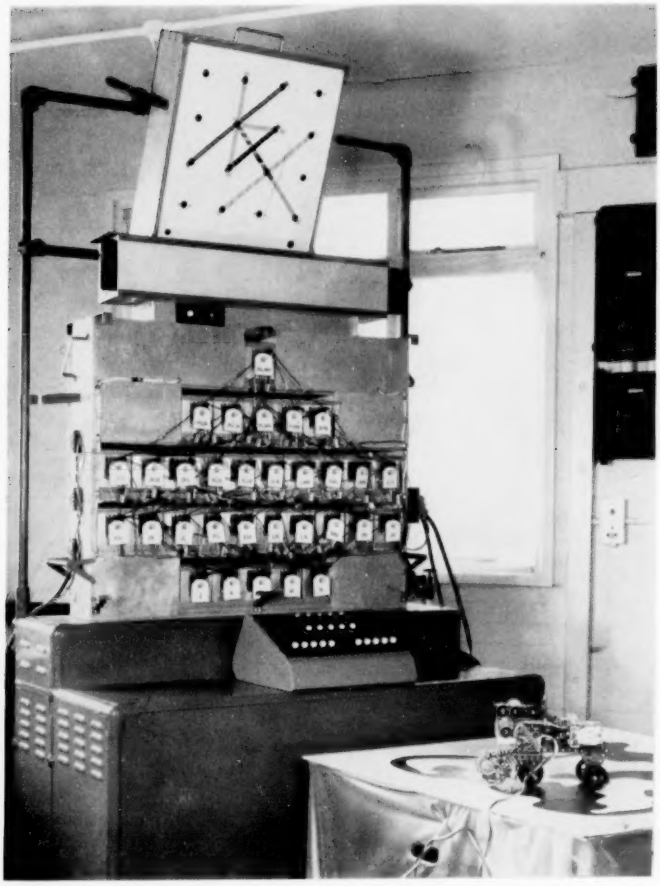

PLATE 1. A conditional probability computer connected to an artificial retina so as to generalize a T-shape, regardless of perspective distortion. The computer can also learn, by trial and error, to control the trolley at the bottom right-hand corner.

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It will be shown that such a system can imitate classical conditioning both as to the development of activity due to coincidences and to their extinction through a lack of reinforcement. Suppose that signals from all exteroceptors, after conversion into a binary form, provide inputs to a conditional probability system; let an unconditioned and a conditioned stimulus activate two sets of inputs S_u and S_c, respectively. In the system there will be two units which distinguish these two patterns and a third unit which distinguishes their conjunction.

Suppose that in the past the patterns S_u and S_c were statistically independent as in the first sequence of eight events in the following table:

	AA'		BB'		CC'		DD'
S_u	01010101	00	11111111	00	11111111	00	0000000000	00
S_c	00110011	11	11111111	11	11111111	11	1111111111	11
$p(S_u/S_c)$		2/4		10/14		18/24		18/36
X	00001111	01	00010000	01	10100101	01	0000000000	01
$p(S_u/X,S_c)$		1/2		2/4		6/9		6/10

For the present, the stimulus X is to be disregarded. If S_c now occurs alone as at A, the conditional probability of S_u is 2/4. If the two stimuli are presented jointly, as in the second sequence, and S_c then occurs alone, as at B, the probability of S_u has risen to 10/14; after a third similar sequence it has risen to 18/24. If the joint presentations continue $p(S_u/S_c)$ will tend to unity (certainty); if S_c then occurs alone the S_u unit will indicate certainty as if S_u had actually occurred; this effect accords with the fact of positive conditioning. The necessary pathway from the S_u unit to the common motor path of the unconditional response will not be discussed at this point.

If, however, the conditioned stimulus is given alone without reinforcement by S_u, as in the fourth sequence, $p(S_u/S_c)$ falls rapidly to 18/36, its original value; this effect corresponds to experimental extinction (internal inhibition). Since $p(S_u/S_c)$ is equal to $p(S_uS_c)/p(S_c)$ it is independent of $p(S_u)$, that is to say, the presentation of S_u alone has no effect on the above results. It is suggested that a similar mechanism underlies the phenomenon of "extinction of meaning". Associations can be built up between pairs of nonsense words; but if one such word is repeated a number of times by the subject it becomes isolated and incapable of evoking any associated words (Wallach, 1955).

In a complicated environment some stimulus patterns will occur at the same time as a key unconditioned stimulus, others will not; so the

conditional probability system may be regarded as a kind of filter (Hick, 1949; Broadbent, 1953) which passes the activity aroused by those stimulus patterns from which S_u may be inferred while it blocks that of others.

It is important to note that although $p(S_u/S_c)=1/2$ at A and at D, the second state of the system is quite different from the first. The number of counts of S_c has risen from 4 to 36, and the number of counts of the conjunction of S_u and S_c has risen from 2 to 18. It is because of this that further effects follow. Suppose that an external stimulus X occurs during the above series of events, as in the above table. The system can compute the probability of S_u given that S_c occurs alone or given that S_c and X are occurring together; the former is the ratio of the counts in the (S_c) and $(S_c\ S_u)$ units; the latter is the ratio of the counts stored in the (S_cX) and (S_cXS_u) units; so the two probabilities are almost unrelated.

In the first sequence X is independent of S_u and S_c; consequently the conditional probability of S_u when S_c occurs at A is the same as at A' when S_c and X occur.

Suppose X occurs once in the second sequence when S_u and S_c are presented jointly; if then S_c occurs as at B, the probability of S_u is 10/14 but if X occurs jointly with S_c, as at B' the probability of S_u drops, since $p(S_u/X,\ S_c)=2/4$. This effect accords with the fact of external inhibition. But as X continues to occur during the third sequence the value of $p(S_u/X,\ S_c)$ increases; so at C when S_c is presented alone the probability of S_u is 18/24, but the occurrence of S_c and X jointly at C' reduces the probability of S_c much less, to 6/9. The inhibitory effect of the external stimulus is much reduced. (The number of presentations of X in the third sequence has been made large to demonstrate this effect without increasing the size of the table.)

Finally, suppose that S_c is presented without reinforcement by S_u as in the fourth sequence. Because $p(S_u/S_c)$ drops to 18/36 the reflex is extinguished; but the joint presentation of S_c and X at D' now increases the probability of S_u to 6/10 in accord with the facts of external disinhibition.

S_u	00111100	11100111	00111100	0000011000000
S_c	11111000	11111000	11111000	0000011111111
X	00011111	00011111	11100000	1111111100000
$p(S_u/S_c)$	3/5	3/5	3/5	1/4
$p(S_u/X)$	3/5	3/5	1/3	1/4
$p(S_u/X,\ S)$	1	0	1/3	2/3

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The above phenomena depend entirely on the arrangement of conjunctions; if they are as in sequence (1) of the above table S_u is conditioned to S_c and to X, and it is more probable if they occur jointly. This is reinforcement and it arises because S_u happens to occur during the joint presentation of S_c and X. On the other hand, in the second sequence, although S_u has been conditioned to S_c and X to the same conditional probability as before, it is less probable if they occur jointly; this arises because S_u happens not to occur during the joint presentation of S_c and X. In this case either stimulus strongly inhibits the effect of the other; it should, therefore, be possible to condition an animal to respond to either of two stimuli when presented alone but to give no response if they are presented jointly. This situation need not be symmetrical or so intense; in sequence three S_u is only weakly conditioned to X which may more properly be called an external inhibitor. In sequence four the S_c–S_u reflex is extinguished by the presentation of S_c without S_u; also S_u has never been fully conditioned to X. Nevertheless, the conditional probability of S_u given that S_c is occurring, is raised if X occurs also. This disinhibition is unaffected by the number of times X occurred before the beginning of the experiment; it is due to the fact that X happens to occur during the phase of positive conditioning to S_c. Sequence four is similar mathematically to sequence one; two weakly conditioned stimuli produce a higher conditional probability when they occur jointly. One concluded that reinforcement and external disinhibition are forms of the same phenomenon; the same view has been expressed by Broadbent (1953.)

2. *Simultaneous pattern completion*

If there has been continuous presentation of S_u and S_c jointly, then when S_c occurs it is, in the system, as if S_u had occurred also. This phenomenon may be described in more general terms as simultaneous pattern completion; it occurs, for example, when an optical image falls partly on the blind spot of a retina; for the subject it is as if the most probable shape has occurred.

At a higher level a written word may be appreciated correctly even if a letter is missing. Suppose that the inputs to a conditional probability system correspond to the letters of the alphabet. If English words are presented for a considerable time the conditional probabilities for all possible sets of letters will be formed in the system. From then on, if a word is presented with a missing letter the system will assess the conditional probabilities of all letters in this context. The system can be designed to indicate any letter whose conditional probability exceed an arbitrary level. This property has been demonstrated practically on

the 5 input machine of Plate 1, using the letters H, E, A, R, D, and a probability threshold of 1/2.

The keys are pressed as follows:

H	. . . 111
E	. . . 1 11
A	. . . 1 1
R	. . . 11
D	. . . 1

to represent the simple sentence "he had her ear". After the sequence has been repeated about eight times the system has computed the statistical structure of this simple language in terms of digram and trigram frequencies. If now the key H is pressed the letter E is inferred because $p(E/H)$ is 2/3; if R is activated E is inferred because $p(E/R)$ is 1 but the system is unable to decide between HER and EAR. On the other hand if H and R are activated E is inferred because $p(E/HR)=1$. Similarly, if D is activated the word HAD is completed because $p(HA/D)$ is unity.

3. *Temporal pattern completion.*

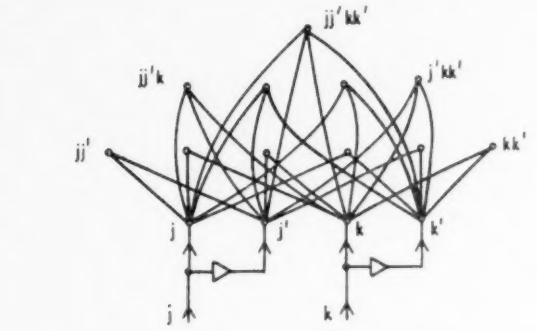
If there are two inputs j and k and the system is to distinguish (classify) only three different temporal patterns

(j before k):	written as $j'\,k$
(j and k simultaneously):	written as jk
(j after k):	written as jk'

then the j input must pass through a delay to form a new signal j'; similarly for k. If the four signals j, j', k, k' form inputs to a conditional probability system as in Fig. 11(a) all the patterns of Fig. 11(b) can be distinguished.

It is most important to distinguish two parts of the system of Fig. 11(a). There is a part which provides delays and nothing else; it supplies signals to the second part, a conditional probability system which analyses only simultaneous sets of signals and computes conditional probabilities simultaneously in negligible time.

Suppose that the pattern (j before k) is presented. First a pulse will arrive at the j input of the conditional probability system and cause a count in the j unit. After a delay a pulse will arrive at the j' input simultaneously with a pulse at the k input; this conjunction will cause counts in the (j'), (k) and ($j'k$) units. Finally there will be a pulse at k' and a count in the k' unit. Let the pattern j before k be repeated a number of times; in the system the quantities $p(k/j')$ and $p(j'/k)$ will tend to unity; it follows that if j' becomes active k will reach certainty; conversely if k becomes active j' will reach certainty. Now let the j



(a)

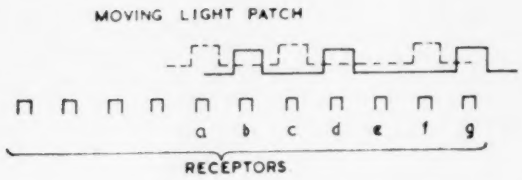
(b)

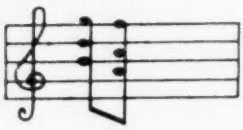
FIG. 11. The classification of spatiotemporal patterns of activity in two binary inputs. (a) A system of coincidence units and delays (shown as triangles) which distinguishes only "before", "simultaneous" and "after". (b) The patterns which can be distinguished by the system.

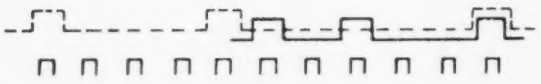
input be activated; after a delay j' will become active and cause k to become certain conditionally. In the system it will be as if the tune (j before k) had been completed. On the other hand, if k is activated, j' will become certain immediately because $p(j'/k)=1$. Had the delay been capable of transmitting pulses in both directions the certainty of j would have produced certainty of j after the delay. Consequently presentation of k would have caused completion of the wrong tune (k before j), thus making nonsense of time's arrow; this effect does not occur if the delays operate only in one direction.

One further example will be considered; suppose that the tune

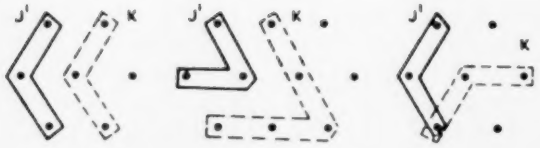
is played. Then, remembering that a dashed letter implies earlier in time, there will be the following conjunctions (jk), ($j'k'$), (jj'), (jk') and ($jj'k'$). If the tune is repeated a number of times there will be equal



(a)

(b)



(c)



(d) (e) (f)

FIG. 12. (a) A moving light pattern on a linear array of receptors.
(b) The spatiotemporal pattern aroused by (a).
Perspective transformations of a light pattern.
(c) Magnification in one dimension.
(d), (e) and (f) Translation, magnification and rotation in two dimensions.

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counts of these conjunctions and the same number of counts of k and k' but there will be twice the number of counts of j and of j'; consequently, $p(j/k)=1$, $p(j/k')=1$, $p(k/j)=1/2$ and $p(j/j')=1/2$. If now the k input is activated, the j unit will reach certainty simultaneously; but after a delay the k' signal will enter the conditional probability system which will infer j a second time—the tune will have been completed. On the other hand, since $p(k/j)=1/2$ and $p(j/j')=1/2$, if j is activated there are equal conditional probabilities for k now and j after; the system is uncertain which j is being played. If the arbitrary threshold for certainty is made a little over 1/2, no tune will be completed. Finally, if both j and k are pressed simultaneously, the tune will be completed; all these facts have been demonstrated with the computer. As it has been pointed out earlier in the paper, degrees of before and after can be distinguished if each input enters a multiple-delay line as in Fig. 3.

The above property of a conditional probability system will be called temporal pattern completion; it is suggested that anticipation in animals is based on a similar mechanism. Being determined by probabilities it must be distinguished from temporal extrapolation which occurs in a gunnery predictor and is determined by time derivatives.

4. *Spatial pattern generalization*

The hypothesis also suggests how the same response might be evoked in a system by different geometrical transformations of the same optical stimulus pattern.

Suppose that a number of light receptors a, b, c, d, e, f, g are placed in a straight line and that each provides an input to a conditional probability system, both directly and via a single delay; let the topology be lost so that any receptor is connected to any input of the conditional probability system. Suppose that an image of some external objects falls on the receptors so as to activate receptors b, d, g, and that the object then moves to a new position such that receptors, a, c, f are activated; this is shown in Fig. 12(a); the conditional probability system receives the spatiotemporal pattern of Fig. 12(b). Let the above event occur frequently; then by temporal pattern completion $p(acf/b'd'g')$ tends to unity and the two stimuli become equivalent; a response conditioned to one will now be aroused by the other. This phenomenon will be called spatial pattern generalization; it has been demonstrated in the model.

By the same means equivalence of stimuli will arise for any repeated transposition (Koffka, 1935) of a stimulus over an array of receptors.

In Fig. 12(c) the stimulus is magnified; in Figs. 12(d), 12(e) and 12(f) the receptors form a two-dimensional array and the transposition of a light patch is shown in translation, magnification and rotation.

In each case if J is the first set of activated receptors and K is the second set, then a consistent change of the pattern from J to K will cause $p(K/J')$ to tend to unity, and the two stimuli to become equivalent.

The fundamental requirement for this phenomenon to occur is not that the topology shall be preserved between the receptors and the inputs to the conditional probability system, but that it shall be preserved between the external stimulus and the receptors; this ensures that the neighbourhood of external objects is represented in the system. It is also necessary that the receptors be connected directly and via delays to the conditional probability system. It is suggested that in such a way spatial pattern generalization occurs in animals—that it is not due to present scanning in the nervous system but to effects stored there which are the result of past scanning by an array of receptors. If this is so the effect must be learnt all over a retina, but approximate fixation will introduce a great economy and minimize this need.

5. *The weighting of past events*

Practical counters possess the limiting states "empty" and "full". Consider a counter in which some quantity is reduced at each count; eventually the counter will reach the limiting empty state. But this would not occur if there were a slow recovery process in the reverse direction and if counting did not exceed some critical rate; in such a counter the effect of a count slowly disappears so that past events are weighted less than recent ones. A counter of this form has been made in a practical computer and it weights past events in an approximately exponential manner. There are at least three consequences of counting in this way. Firstly, the number of conjunctions of A and B which are required to raise $p(A/B)$ above some threshold level depends on the time intervals between the conjunctions; this number can be calculated from a knowledge of the weighting function of the counter. Secondly, the effects of old events eventually disappear from the system so that conditional probabilities can always be modified by new events. Thirdly, if no events occur for a considerable period, the recovery process will cause all counters to reach the full state; this will cause all conditional probabilities to become unity—an intolerable state of affairs in which anything implies everything. There seems to be no solution to this problem other than to ensure that there never is such a period of no events; this can be achieved if, in the absence of real events, each input becomes active spontaneously in a random manner which is independent of that of other inputs. If the mean random firing rate is low compared with that of the occurrence of external events it

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will have little effect on computed conditional probabilities; but when such events cease the random background activity will slowly destroy any inferences which may have been formed.

6. *Spontaneous recovery*

If, after a long period of conditioning an animal, the reflex is rapidly extinguished by non-reinforcement of the unconditioned stimulus, the reflex will then recover spontaneously without further conditioning. This means that the events which occurred during the period of extinction are, for a while, given enhanced weight in comparison with the earlier ones, but that as they sink into the past all events tend to have the same relative weighting. With an exponential weighting function the counts of all events, recent and past, are scaled down in exactly the same way with the passage of time. A counter which will introduce the phenomenon of spontaneous recovery must possess a weighting function which gives even greater weight to recent events than does an exponential function. Such counters have been made; they do demonstrate spontaneous recovery.

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IV. Conditional Probability Computers

Computors have been built which demonstrate all the forms of behaviour which have been described in the above section; their detailed design has been discussed elsewhere (Uttley, 1959a).

A conditional probability computer consists of a large number of identical units connected in a lattice as for a classification system. The additional need for counting, i.e. storage, on an approximately logarithmic scale is met by two additional properties of each unit.

(1) When any unit is not indicating occurrence, some physical quantity associated with it grows—preferably in an approximately exponential manner.

(2) When a unit indicates occurrence this physical quantity is decreased, preferably by a fairly constant fraction of its present value. Such a reversible process occurs frequently in living tissue.

1. *Supercontrol*

The requirement here is that when a pattern of activity a occurs, the a unit shall count and cause to be emitted from every superunit ab a signal depending on $p(b/a)$. With the arbitrary choice already made of a positive logarithmic scale of representation, the emitted signal must be proportional to the logarithm of the quantity stored in the superunit minus the logarithm of the quantity stored in the counting unit. It follows that the output of a superunit is increased if the count in it

increases and is decreased if the count is increased in the subunit which is affecting it. So two rules follow:

(3) If a unit counts, its effect on superunits is reduced, i.e. it becomes a less sensitive transmitter.

(4) If a unit counts, the effect of counting subunits on it is increased, i.e. it becomes a more sensitive receiver.

They can be interpreted as demanded laws of synaptic transmission (Uttley, 1959b). A probability is the ratio of two numbers and the probability theory suggests that the numerator is stored the receiving side of a synapse and the denominator on the transmitting side.

Perhaps the most important point to be noted at this stage is that, according to the probability theory, the effect of one neuron on another depends on their relative state, i.e. on two independent variables, whereas the facilitation theory refers to only one variable. The following purely physiological argument supports the suggestion that one variable is inadequate to describe the past history of a synapse. Consider two neurons T and R for which an axon of T makes functional connexion with a dendrite of R. If T and R are part of a richly interconnected population of neurons there will be occasions when T fires but R does not, because of inadequate additional excitation from its connected neighbours. Conversely, with sufficient excitation from its other neighbours R may fire when T does not. It follows that the two sides of the TR synapse must have different past histories.

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2. *Difficulties of the hypothesis*

Although the theory explains many forms of animal behaviour there are a number of physiological difficulties regarding its functioning. Firstly, the requirements that, after firing, a neuron shall become a less sensitive transmitter and a more sensitive receiver are the reverse, respectively, of post-tetanic potentiation and adaptation. This difficulty can be met by reversing the scale upon which probabilities are represented to a negative logarithmic one. Outputs due to events are then strictly proportional to their information gain so that there is a large output for the unpredicted event.

The above rules (3) and (4) for supercontrol are then changed and, in neural terms, become:

(3a) After a neuron has fired it becomes a more powerful transmitter at all its axonal synapses (post-tetanic potentiation).

(4a) After a neuron has fired it becomes a less sensitive receiver at all its dendritic synapses (adaptation).

It can be seen that, in such a neural system, experimental findings could easily seem to be conflicting, some supporting facilitation—

some adaptation, if the changes in state of a synapse were thought of in terms of one variable rather than two.

The negative logarithm of probability will be called *Rarity* and the changed system a Conditional Rarity Computer. In such a system an engineering inelegance disappears. Suppose that patterns a, b and c nearly always occur together. Then, in a Conditional Probability Computer if input a occurs alone there will be large outputs from the ab, ac and abc units; this is hardly an economy of channel capacity. In a Conditional Rarity Computer only unit a will become active. Consider a further situation; suppose that a occurs often in context b and rarely in context c. Then if a and b occur the ab unit will count but its output will not be very active; but if a and c occur the ac unit will count and give a highly active output. If a occurs alone it will have little effect on the ab unit but a big effect on the ac unit; however, the ab and ac units will not count.

A second difficulty in a Conditional Probability Computer is that each unit must become active in two different ways—when counting occurrences and when indicating probable inferences. The latter activity must not cause counting, i.e. change the state of a unit.

The same difficulty arises in a Conditional Rarity Computer if it is to perform the two functions of:

(1) Developing signals proportional to the information gained by events which occur.

(2) When events occur, developing signals proportional to the information gained for all events which do not occur.

If the latter function is left out then units become active in only one way, by actual occurrence of corresponding events; this activity, though proportional to information gained, always causes counting in the units.

It is now necessary to return to the problem, considered earlier in the paper, of discrimination in a system with a number of neurons which is small compared with 2^n the number of possible patterns of activity in n inputs. The suggestion was made that neurons should be overcritically connected initially, with ambiguity of classification, and that adaptation should consist of removing connexions which imply high probability. This system would be an overconnected Conditional Rarity Computer and its properties will now be discussed briefly.

V. Over-connected Conditional Rarity Systems

Consider, first, a classification system consisting of a large number of neurons, each with a fixed threshold, i.e. such that it will fire if more than a small fixed number, r, of its inputs are active. Suppose also that each neuron has R inputs where R is much greater than r. The number

of possible input patterns to each unit is 2^R and a negligible number of these—those with less than r elements—will fail to fire the neuron. The ambiguity of discrimination of each unit may be said to be about 2^R.

If there are N such neurons there will never be more than N distinguishable patterns even if variation of threshold is introduced; but, in principle, such a system should be capable of adapting itself to discriminate N patterns from a much wider group of $N \times 2^R$ patterns. This number could be a sizeable fraction of the total number of 2^n patterns which could arise if there were a total of n inputs to the entire system.

Suppose, now, that a neuron is given the chosen property of being a more powerful (axonal) transmitter and less sensitive (dendritic) receiver after firing. Suppose that, of all the 2^R sets of inputs which might occur at the input to the neuron, one set occurs far more often. Then these axons will become more powerful relative to the others and as the neuron becomes less sensitive only they will fire it. The ambiguity will have been resolved.

However, the system here described is one only for classifying or discriminating input signals in a plastic manner which adapts to the past experience; it makes no inferences. If inference is also demanded, and this seems essential in trial-and-error learning, the physiological difficulty returns of a neuron indicating in two different ways—for occurrence and for high inferred probability. The difficulty might be resolved by carrying out classification and inference in different parts of the total nervous system.

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VI. The Discrimination of Continuously Variable Signals

The difficulty of discriminating continuous signals describable in terms of more than three variables was described at the beginning of the paper. Nevertheless, some such discrimination is possible. An animal can differentiate tones of similar frequency. A man can distinguish speech sounds which require some six or seven variables for their description.

Andrew (1959), Barlow (1959) and Selfridge (1959) have proposed that, to obtain some measure of similarity of two variable signals, one should compute their coefficient of cross-correlation. This is the product of the two signals, integrated over the past, with some weighting (forgetting) function and, perhaps, with some scaling factor depending on the separate signals. This process closely resembles the weighted counting of coincidences of binary signals which is needed to determine a conditional probability. It is interesting to note that, if the two variables are signalled with a frequency-modulation code—as occurs in

the nervous system—then a simple coincidence counter with memory does compute the cross-correlation coefficient. Andrew (1959) has developed a trial-and-error learning system using cross-correlation; the type of learning of which it is capable resembles the acquisition of motor skill much more than that of, say, maze learning. It is a system for learning *how much* force, say, is required in a given context, rather than for making binary or even multi-choice decisions. Selfridge has done similar work independently. Barlow (1959) has shown that, by cross-correlation methods, continuously variable signals in two channels can be coded automatically into signals demanding smaller channel capacity (bandwidth) if there is some statistical dependence between them.

VII. CONCLUSIONS

A system of richly interconnected units of variable sensitivity and sending signals to one another of variable strength would be capable of extremely subtle behaviour. This could be of very wide variety depending on the exact laws governing the effect of past events on connectivity, signal strength and unit sensitivity. It might well be that nothing more is needed, physically, to explain animal and human behaviour. The study of such a system demands, *inter alia*, the training of mathematicians and control engineers.

If incoming signals are binary the system can behave as a conditional probability computer, using an approximately negative logarithmic scale. It is necessary that each unit, after firing, become a more powerful emitter of signals and a less sensitive receiver; this is a physiological theory which can be tested. Such a conditional probability system can imitate many forms of learning which result in binary or multiple choice. Such a system is also capable of pattern generalization and classification. It can distinguish only as many classes of signals as there are units in the system; but if N units each possess R connexions to neighbours the system can adapt or learn to discriminate N frequently occurring patterns from $N \times 2^R$ possible patterns; this might be a very large number.

If the incoming signals are continuously variable and represented by a pulse-frequency code the same system can compute coefficients of cross-correlation between signals. By this means it can behave as a plastic coding system for reducing redundancy between signals and so making more effective use of its channel capacity. The same system is capable of quantitative learning, e.g. of deciding how much force should be produced in a given context in a motor skill.

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3

EFFECTS OF X-RAYS ON NUCLEIC ACID BIOSYNTHESIS AND ON THE ACTIVITY OF NUCLEASES IN MAMMALIAN CELLS

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EFFECTS OF X-RAYS ON NUCLEIC ACID BIOSYNTHESIS AND ON THE ACTIVITY OF NUCLEASES IN MAMMALIAN CELLS

R. Goutier

I. Nucleic Acids

Many publications deal with the alterations of biosynthesis of nucleic acids in tissues by ionizing radiations, because the understanding of that lesion is of evident importance in radiobiology. Excellent reviews have recently been published on that subject: besides the very thorough monograph of Errera (1957) and the less recent book of Bacq and Alexander (1955), let us mention the articles of Howard (1956), Kelly (1957), Holmes (1957) and Stocken (1959). We only wish to discuss here a few topics of the broader subject.

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1. *Late effects* in vivo

After irradiating a whole mammal, biochemists generally follow the variations of two variables: the nucleic acid (NA) content or concentration in tissues and the rate of their biosynthesis, measured by the incorporation rate of small molecules into NA. This last variable seems to be the earliest sensitive one to ionizing radiations, since it decreases almost immediately after irradiation while the deoxyribonucleic acid (DNA) content remains normal. Ord and Stocken (1956) observed that ^{32}P incorporation into rat thymus DNA was inhibited by 50 per cent, 3 min after 1000 r total-body X-irradiation. Within 1 hr after 100–800 r total-body irradiation of rats, Nygaard and Potter (1959) found an inhibition of ^{14}C-thymidine incorporation into thymus and spleen DNA, the depth of inhibition depending on the irradiation dose. In the small intestine, 200–800 r also produce a similar inhibition within the first hour.

Such observations are not frequent: most of the publications describe observations performed several hours or days after irradiation.

For all articles published before 1957, the reader may refer to the above-quoted reviews. Only the more recent publications will be considered here.

Harrington *et al.* (1957) measured the ^{32}P incorporation into DNA of various mouse tumours. Three hours after 1000 r, incorporation rate

is only 65 per cent of normal in the Ehrlich ascites tumour; it is very little affected by 2000 r in 6C3HED lymphosarcoma and not at all by 5000 r in the Lettré hyperdiploid Ehrlich tumour or in TA3 adenocarcinoma. These are two tumours in which DNA synthesis is rather radioresistant despite their high rate of proliferation.

In mouse spleen, 2 hr after 900 r, ^{32}P incorporation into DNA is decreased by 59 per cent (Hagen, 1958). According to Mole and Temple (1959), a decrease of deoxyribonucleoprotein (DNAP) content in the small intestine of the mouse can only be obtained 12 hr after a single dose of 1000 r; the depth of that decrease is directly related to the dose, with the exception of the dose of 25,000 r which is less efficient than 5000 r. The deeper the depression of DNA content, the later the beginning of the recovery. Incorporation of ^{3}H-thymidine, however, measured by autoradiography in mouse intestine (Sherman and Quastler, 1958) is affected much earlier and by smaller doses than DNA phosphorus content: 3000 r reduce to one half in 15 min the incorporation rate of thymidine and abolish it in 2 hr X-ray doses down to 200 r have a similar but slower effect. As pointed out by Kelly (1957), the cells synthesizing DNA in the small intestine are located in the glandular crypts and represent only a small proportion of the total intestinal cell population. Since those cells are selectively affected by irradiation, one cannot expect a marked decrease in total DNA content which is mostly inert DNA.

In spleen of rats given 325, 500 and 700 r, Mandel and Chambon (1959) observed an immediate decrease in the DNA content which, on the fourth day, is only 30 to 35 per cent of the normal value after any X-ray dose. The recovery occurs more rapidly after the smaller dose (325 r) and the normal DNA content is reached again on the fourteenth day. The relative specific activity of DNA (measured 12 hr after injection of ^{32}P) is 30 per cent of normal 2·5 days after irradiation and recovers its normal value on the seventh day, preceding, as might be expected, the alterations of the DNA content.

Nygaard and Potter (1959) followed during 24 hr the variations of the specific and total activities of DNA thymine in rat spleen, thymus and small intestine. All the doses employed, from 50 to 800 r, produce a decrease in ^{14}C-thymidine incorporation. After the smallest dose (50 r) however, the decrease is preceded by a 15 per cent increase of the specific and total radioactivities in spleen and small intestine during the first hour, and a 10 per cent increase in thymus during the second hour. In the small intestine, the minimum is reached 8 hr after any dose: it represents 60 per cent of the normal value after 50 r and 14 per cent after 800 r. Incorporation rises thereafter more or less rapidly according to the X-ray doses administered; the total activity recovers its normal

level 24 hr after 50 and 100 r, but remains very low (18 per cent normal) 24 hr after 800 r.

In Nygaard and Potter's experiments (1959) there are two difficulties which are often encountered in similar works. The first one is common in the use of tracers. Nygaard and Potter observe in the thymus a thymidine incorporation rate hardly higher than in the liver. Since mitotic index is much higher in the former tissue than in the latter, there is, in this case, a discrepancy between synthesis and incorporation rate. The authors offer an explanation : viz. by increasing the amount of carrier, they do not alter the incorporation rate in the thymus, a fact which points to the existence of a large pool of precursors in the thymus and, consequently, to a dilution of the tracer. This does not seem to be the case in spleen or in the intestine.

The second difficulty is the interference of dying or dead cells, inevitably present in radiosensitive tissues later than 1 hr after irradiation, lowering by their inert DNA the values of the specific activity. When those cells and cell debris are cleared away, the specific activity rises, but the total activity keeps constant so long as there appears no healthy proliferating cells. This is indeed what Nygaard and Potter observe in the spleen.

After total-body irradiation of a mammal, two factors interfere with the observation of DNA synthesis, besides any artefact due to the tracer technique: the changes in cell population and the abscopal effects (according to Mole's definition, 1953). We would like in the first instance to comment upon these two topics.

2. *Changes in cell population and abscopal effects*

The changes occurring in the cell population of a tissue must be taken into account when nucleic acid metabolism is studied later than 2–3 hr after irradiation. This question has already been discussed by Howard (1956) and Kelly (1957), who give excellent examples of the influence of these changes on the ^{32}P incorporation rate in various tissues. According to these authors, the inhibition of DNA biosynthesis in spleen, thymus and small intestine may actually be explained solely by the changes in cell population. This conclusion is still valid for many instances of long-term observations after irradiation, but in view of the very early effects which will be described later on, we think that some restriction should be made to its generalization.

If the first change is the appearance of dead cells or cell debris, another ulterior change may also be important; after the cell debris have been cleared away, healthy cells recolonize the damaged tissue and may often be different from the cells initially present before irradiation. Biagini *et al.* (1958) isolated nuclei from rat lymph node

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cells after total-body irradiation: cells reappearing a few days after irradiation had a larger nucleus, a lower DNA content and a lower ^{32}P incorporation rate into DNA than normal cells. Cooper (1959) observed that 300 r applied to a rat brought about a decrease in the percentage of large lymphocytes in the lymph, but enhanced the proportion of those lymphocytes synthesizing DNA.

One cannot deny the importance of a factor which has already been discussed by others (Errera, 1957; Howard, 1956; Kelly, 1957). It concerns the necessity of histological controls for all biochemical measurements performed with a long delay after irradiation. In this respect, Lajtha (1958) has recently emphasized the advantages of autoradiography, which scans a tissue cell by cell, over measurements of specific activities of nucleic acids, which are quantitatively more accurate but blinder.

By making observations less than 1 hr or $\frac{1}{2}$ hr after irradiation, biochemists avoid criticism as regards changes in cell population, but are still exposed, to some extent, to the influence of abscopal effects such as those described below.

Berenbom and Peters (1956) found that, 2–7 days after 400 r total-body X-irradiation, rat spleen DNA, while strongly decreased in amount, displayed an enchancement of the base ratio purines/pyrimidines which, starting from 0·94 in normal animals, went up to 1·9 in the irradiated ones. After 400 r local irradiation of the exteriorized spleen, the base ratio remained normal and the DNA content, not so deeply depressed, recovered its normal value more rapidly than after total-body irradiation (Berenbom, 1956; Petersen *et al.*, 1955). De Hevesy (1945) showed that in a rat bearing two tumours, the irradiation of one of them altered the ^{32}P uptake by the second. From similar experiments made by Holmes (1949), it appears that DNA content decreases by 75 per cent in the irradiated tumour and by 50 per cent in the protected one.

Kelly and Jones (1950) described a drop in DNA turnover in a rat mammary tumour after selective irradiation of liver or muscle (carried out by, respectively, intravenous or intramuscular injection of yttrium). More recently, Harrington and Lavik (1956, 1957) studied the ^{32}P incorporation into mouse Ehrlich tumour DNA by varying the coupling of *in vivo* and *in vitro* conditions of irradiation and incubation. No inhibition of incorporation is observed if (1) the tumour from a total-body irradiated animal is incubated *in vitro* with ^{32}P, or if (2) tumour cells are irradiated and incubated *in vitro* with ^{32}P, or if (3) normal tumour cells, previously incubated with ^{32}P, are injected into irradiated mice. Inhibition of ^{32}P uptake by the tumour only occurred when (1) irradiation and incubation take place *in vivo* or (2) when cells, incubated

in vitro with ^{32}P, are irradiated before being injected to previously irradiated mice.

By irradiating (800 r) rabbits with one longitudinal half of the body protected by a lead shield, Uyeki *et al.* (1959) produce, 24 to 48 hr after irradiation, a drop in the number of cells of high ploidy, not only in the marrow of the irradiated leg, but also, less-markedly, in the protected leg.

Despite the investigations proving their existence (see also Casarett, 1958; Shapiro, 1957a, b, 1958), the humoral factors responsible for those abscopal effects are still unknown.

Reciprocally, non-irradiated protected tissues exert a protective and restoring action on irradiated tissues. Albert (1958) observes numerous mitotic abnormalities in regenerating mouse liver only when other parts of the body are irradiated at the same time as the liver. Rat spleen is less damaged by local irradiation than by total-body irradiation: 1000 r *in loco* are necessary to produce histological lesions and a 5 per cent drop of weight in 5 days (Boiron *et al.*, 1955). Given to the exteriorized liver, 10,000 r do not produce any histological lesion, nor do they impede the ulterior regeneration if two out of three lobes are cut out immediately after irradiation (Gershbein, 1956).

The conclusion to be drawn from those examples is that in a total-body irradiated mammal, the alterations of many biochemical functions observed in a particular tissue originate in most cases from a mixture of effects of the absorption of radiation and of abscopal effects.

3. *Radiosensitivity of the presynthetic period (G_1) in the mitotic cycle*

From the experiments of Howard and Pelc (1953), it is known that the mitotic cycle of many types of cells can be divided into 4 periods: the actual mitosis is followed by a G_1 period during which the DNA remains constant; then comes the synthetic period where the DNA content is doubled; this latter period is separated from the next mitosis by a G_2 period.

In his review, Stocken (1959) notices that DNA synthesis is inhibited by much lower doses of X-rays when the cells are irradiated during the G_1 period than during the synthetic period.

Among mammalian tissues, rat regenerating liver is a good material, because it is one of the rare cases where there exists, *in vivo*, a synchronism of DNA synthesis and of the first waves of mitoses between the cells. Holmes (1956) has described the evolution of ^{32}P incorporation into DNA and of mitotic index after partial hepatectomy. DNA synthesis takes place from the eighteenth to the thirtieth hour, with a maximum at the twenty-seventh hour. The mitotic index increases considerably and reaches a maximum at about 30 hr. Relatively weak

doses (375 r) given before the beginning of DNA synthesis inhibit orotic acid incorporation into DNA; 1500 r suppress it. But as soon as synthesis has started, 1500 r have hardly any effect (Beltz *et al.*, 1957). Kelly *et al.* (1957) induce regeneration by poisoning mice with CCl_4. In this case, the time-course of the phenomena is not quite the same as after surgical hepatectomy: the maximal DNA specific activity is only reached after 40 hr and mitotic activity is observed from the forty-fourth hour onwards. Here, too, 800 r total-body irradiation delay the DNA synthesis provided they are given before the onset of synthesis, that is at the twelfth, seventy-second and ninety-sixth hours after CCl_4 treatment. But 2000 r are almost harmless when administered during the DNA synthesis, between the twenty-fourth and forty-eighth hours. When regeneration is induced by CCl_4 poisoning, the effects of irradiation are less pronounced than after partial hepatectomy: as suggested by Kelly (1957) it is possible that anoxia, due to CCl_4, might lower the radiosensitivity of the liver.

The interesting experiments of Lajtha *et al.* (1958) have extended those results to cultures of human bone-marrow cells. Incubated with ^{14}C-formate, bone-marrow cells incorporate 95 per cent of ^{14}C into the methyl group of thymine. By incubating with the labelled precursor for 4 or 5 hr after irradiation, all the cells labelled will have been in the synthetic period (S) during irradiation. If the label is added between 17 and 22 hr after irradiation, then all the labelled cells will have been irradiated while being in the G_1 (presynthetic) period. Lajtha *et al.* (1958a, b,) measure the incorporation rate by autoradiography and observe that 200–300 rads given to cells in S do neither inhibit nor delay DNA synthesis, but if administered to cells in G_1, they prevent half of the total number of cells to enter the S period at a normal time. The synthesis is only *delayed:* entering the S period after some delay, the irradiated cells synthesize DNA at a normal rate. Whether the culture be irradiated *in vitro* or the sternum is irradiated locally *in vivo* before puncturing the marrow cells, 400 r given in G_1 delay DNA synthesis to the same extent in both cases. Much higher doses are required to decrease the ^{14}C-formate incorporation rate in synthesizing cells: 2000 r slow down only by half the rate of DNA synthesis and one must give as much as 10,000 r to observe a 75 per cent inhibition (Lajtha *et al.*, 1958a).

According to Paul (personal communication, 1959), ^{3}H-thymidine incorporation into DNA of fibroblasts *in vitro* is inhibited only when the cells are irradiated immediately after their inoculation to the medium, that is, when most of them are in G_1. Performed 24 hr after inoculation, irradiation is much less effective.

Lajtha considers that there exists in G_1 a mechanism which is more radiosensitive than DNA synthesis itself.

This phenomenon, however, does not seem to be met with in any type of cells in culture. Painter and Robertson (1959) incubated with ^{3}H-thymidine HeLaS3 cells previously irradiated with 500 r: the proportion of labelled cells in the autoradiograms was higher than in the controls. Since the mitotic index was deeply depressed, one must admit that the slowing down of the mitotic cycle has taken place in the S period and not in G_1; staying longer in S, more cells will be labelled at a given time. Unfortunately, Painter and Robertson have probably lost the synchronism of the mitoses because they start observations only 3 days after inoculation.

4. *Radiosensitivity of the DNA synthesis period*

There is still controversy on the question whether radiations have a specific action on DNA biosynthesis or whether the observed inhibition of synthesis could not be solely accounted for (1) by the mitotic arrest and the consequent prevention of cells to enter the next S period and (2) by the contamination by dead cells. This second point has already been mentioned before. It must be considered in long-term experiments after irradiation but is not relevant in observations made immediately after irradiation, for example, in Ord and Stocken's observation (1957) that within 3 min, 1000 r produce a 50 per cent inhibition of ^{32}P incorporation into rat thymus DNA.

Kelly *et al.* (1957) did not find any decrease in ^{32}P incorporation rate into ascites tumour DNA in the course of 1 day after 800 r: mitoses are blocked, but cells synthesize DNA at a normal rate until the premitotic value is reached. To interpret these results, one must remember that according to Hornsey and Howard (1956), the mitotic cycle of the Ehrlich ascites tumour cell is divided into three parts instead of four. Mitosis is succeeded by the S period, without intermediate G_1 period. Since DNA synthesis in regenerating liver and bone-marrow cells was inhibited or delayed when the irradiation was performed preferably in the G_1 period, one may expect a lesser efficiency of irradiation on ascites tumour cells where the G_1 period is lacking.

According to Forssberg and Klein (1954), however, 1200 r, a higher dose than that used by Kelly, bring about a 30 per cent inhibition of ^{14}C-glycine incorporation into ascites tumour DNA. Administered to Ehrlich ascites tumour cells *in vitro*, 3000 r also inhibit the ^{14}C-uracil uptake by DNA thymine; this inhibition is more pronounced when growth is stimulated by addition of serum to the incubation medium (Harbers and Heidelberger, 1959).

In the Yoshida sarcoma of the rat, 1000 r *in vivo* block the mitoses and prevent any increase in DNA content (Gardella and Lichtler, 1955).

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After total-body irradiation, the specific activity of the DNA of the resting liver decreases: according to Kelly (1958), this cannot be explained either by reduction of food intake, or lengthening of mitotic time—which is already very long in normal liver—or by cell death which is undetectable.

In Earle's L cells and monkey kidney cells *in vitro*, Whitmore *et al.* (1958) observe that after 5000 r, ^{32}P incorporation into DNA goes on for 6 days, though at a half or one-third of the normal rate. Logan *et al.* (1959) followed by autoradiography the ^{14}C-adenine incorporation into NA of isolated thymus and liver nuclei. Two hours after 300 r, incorporation is 50 per cent of normal in DNA of liver nuclei, whereas it is normal even after 900 r in thymus nuclei. These latter nuclei do not synthesize DNA *in vitro:* the incorporation measured corresponds to a turnover and not to a synthesis; the former is less radiosensitive than the latter.

^{32}P incorporation into rat lymph nodes DNA is depressed by irradiation within 2 hr to 70 per cent of normal *in vitro* and to 40 per cent *in vivo* (Ord and Stocken, 1956). ^{32}P incorporation into DNA of rabbit appendix cells is inhibited by 500 r *in vitro;* after that dose, the inhibition is less marked in the isolated nuclei of the same cells (Yuong-Tchel Tchoe and Sibatani, 1959).

In rat thymus, inhibition of incorporation into DNA *in vivo* can be detected after 25 r and is as high as 49 per cent of the control values 3 min after 1000 r (Ord and Stocken, 1956).

Van Lancker (1959) irradiated rats 24 hr after partial hepatectomy, that is during the DNA synthesis period, and observed that 6 hr after 1500 r, the incorporation of ^{3}H-thymidine into DNA of homogenates was depressed. The same is true for DNA of mouse thymus homogenates 6 hr after 350 and 700 r.

According to Sherman and Quastler (1958), doses from 200 to 3000 r not only delay the onset of synthesis in resting cells, but also affect cells in the synthetic period. Incorporation of thymidine into DNA of mouse hair follicles is already inhibited 20 min after 800 and 3000 rads: this effect occurs too early to be accounted for by mitotic arrest or by cell death (Cattaneo *et al.*, 1959).

The evidence thus points to inhibition of DNA synthesis already in progress by relatively high X-ray doses. Doses below 1000 r, in certain tissues, only delay the onset of synthesis. A slight enhancement in rate of synthesis has even been observed by Nygaard and Potter (1959) for doses up to 100 r. In his review, Errera (1957) quotes other examples of synthesis acceleration.

In a recent communication, Nygaard (1959) notes that ^{14}C-thymidine incorporation into spleen and small intestine DNA is inhibited

immediately after doses ranging from 100 to 4600 reps. According to this author, all doses have an inhibitory action.

Mitosis cannot occur if the cell is no longer capable of synthesizing DNA. Many authors, however, doubt that the antimitotic action of X-rays can be explained by the inhibition of DNA synthesis. Many think that mitotic arrest is not related at all to the slowing down of DNA synthesis. In some cases, it is possible to dissociate the two phenomena.

It is possible to block mitoses without impeding DNA synthesis, as observed in ascites tumour, with medium X-ray doses, by Kelly *et al.* (1957), and in Earle's L cells *in vitro* by Whitmore *et al.* (1958).

Doses of 450 r delay mitoses in regenerating liver but are almost ineffective on DNA synthesis when given during the synthesis (Holmes, 1956).

Administered to fibroblasts *in vitro*, 500 r lower the mitotic index without affecting the DNA synthesis to the same extent (Harrington and Smith, 1959).

We may also quote the following examples from micro-organisms:

(1) By cyclic variations of the incubation temperature of *Tetrahymena pyriformis*, Ducoff (1956) causes the cells to synthesize DNA without dividing. On returning to normal temperature, the cells divide several times in succession without DNA synthesis. After irradiation, the mitotic inhibition is the same for any value of the cellular DNA content.

(2) Yeast cells continuously irradiated for 2, 4 or 6 hr at a rate of 3·9 kr/hr stop dividing but go on enlarging without decrease in DNA content (Spaerl and Looney, 1958). After 10^5 r, the budding of yeast cells is abolished whereas ^{14}C-uracil incorporation into DNA thymine is only partially inhibited (Chantrenne and Devreux, 1959).

It may be concluded that mitosis and DNA biosynthesis may be affected independently by X-irradiation. This conclusion, however, is drawn with the implicit assumption that incorporation of labelled precursors takes place only during the synthesis of new DNA molecules prior to mitosis and that there is no metabolic turnover. At the present time, most of the evidence is in favour of the stability of DNA in non-proliferating cells (Brown and Roll, 1955; Kihara *et al.*, 1956; Sibatani, 1957). Recently, in non-dividing rabbit macrophages *in vitro*, Watts and Harris (1959) did not detect any incorporation of thymidine or adenine into DNA.

Other observations, however, seem to question the concept of stability of DNA. According to Paul (personal communication, 1959), giant fibroblasts, obtained after irradiation *in vitro*, still incorporate thymidine into their DNA, a fact which is considered to be due to a

turnover. The incorporation rate of ^{3}H-thymidine and adenine into DNA of mouse seminal vesicles corresponds to a daily renewal of 10 per cent of the total DNA: this figure is much superior to what one would expect from the mitotic index, and compels one to admit the existence of metabolic activity in DNA (Pelc and Gahan, 1959). Although the evidence in this direction is still scanty, it is not impossible that this fact may have to be taken into account in the future. (See also Thomson *et al.*, 1958.)

5. *Radiosensitivity of ribonucleic acid synthesis*

The effects of X-irradiation on both types of nucleic acids are not always similar. In general, ribonucleic acid (RNA) synthesis is less sensitive to radiation *in vivo*, as detailed by Errera (1957). Cytoplasmic and nuclear RNA behave differently because, in a normal cell, RNA metabolism is more active in the nucleus than in the cytoplasm (see references in Smellie, 1955).

Harbers and Heidelberger (1959) observe that *in vitro* irradiation of ascites tumour cells by doses ranging from 750 to 3000 r inhibit to 75–50 per cent of normal the uracil uptake into nuclear RNA without altering the uptake into cytoplasmic RNA. After 3000 r, thymidine incorporation into DNA is inhibited to the same extent as uracil uptake by nuclear RNA.

In regenerating liver, incorporation of orotic acid into cytoplasmic RNA is not affected by 1500 r; unfortunately, the authors did not attempt to separate nuclear RNA (Beltz *et al.*, 1957).

Mandel and Chambon (1959) studied the variations of total RNA content and ^{32}P uptake by RNA in spleens of rats irradiated with 700 r from 1 to 21 days previously. In this case, the alterations of RNA are closely similar to those of DNA: the minimum value of the RNA content is reached on the seventh day, that of its relative specific activity is observed after 2·5 days. But the continuous change in the cellular composition of the spleen during the observation period complicates the interpretation of these results.

After 5000 r *in vitro*, ^{32}P uptake by RNA and DNA of Earle's L cells is inhibited to the same extent (Whitmore *et al.*, 1958).

In isolated nuclei, where mitosis no longer occurs, it seems even possible to inhibit RNA renewal more than DNA synthesis. Logan *et al.* (1959) irradiated nuclei of thymocytes (50, 300 and 900 r) and found, after all doses, the same inhibition of adenine uptake by RNA without any decrease of uptake by DNA.

A similar result is obtained by Yuong-Tchel Tchoe and Sibatani (1959) with nuclei from rabbit appendix cells: 580 r lower ^{32}P incorporation by 13 per cent into DNA and by 59 per cent into RNA.

Thus, contrary to measurements *in vivo*, incorporation rates measured *in vitro* reveal that RNA synthesis is no less sensitive to irradiation than DNA synthesis. It is possible that the relatively lower sensitivity of cytoplasmic RNA compared with nuclear RNA may be due to its location and to the closer vicinity of mitochondrial energy sources.

6. *Mechanisms of inhibition of biosynthesis of nucleic acids*

Errera (1957) and Stocken (1959) lay stress on the accumulation of nucleotides after irradiation and conclude that X-rays do not interfere with nucleotides synthesis but inhibit their polymerization into nucleic acids. According to Forssberg and Klein (1954) adenosine triphosphate (ATP) synthesis is not altered in Ehrlich ascites cells after 1200 r. Bishop and Davidson (1957) observe an increase of deoxyribonucleotides in rabbit appendix and thymus 1 hr after 1000 r.

Mandel and Chambon (1959) described the variations of ribonucleotides content and relative specific activity in rat spleen 1–21 days after 700 r. After 3 weeks, the total amount of spleen nucleotides is the same in irradiated animals as in the controls, but at any earlier date, it is much higher in the irradiated group. Although the amount varies, the relative specific activity of the ribonucleotides remains constant: that proves that they are synthesized at a normal rate. Mandel and Chambon observed a rise in the content of deoxyribonucleotides of an ascitic hepatoma 12 hr after irradiation; they infer, by analogy, that the same phenomenon might occur in the spleen, but in view of the difficulty of determination of deoxyribonucleotides in spleen, they present no direct evidence. Moreover, degradation products from dead cells DNA would confuse the picture in spleen (Main *et al.*, 1957; Cole and Ellis, 1957). At the twenty-first day, the amount of spleen nucleotide triphosphates (the true precursors of nucleic acids) is 1·5 times higher than normally; nucleotide mono- and diphosphates are below normal level; at that moment, DNA synthesis is four times more active than in the control group.

In rat thymus, 1 hr after 1000 r whole-body irradiation, Ord and Stocken (1958b) observe an accumulation of deoxyribonucleotide mono- and triphosphates (particularly deoxycytidine and deoxyuridine mono- and triphosphates). No thymine nucleotide is detectable, but deoxyuridine phosphate, which is not a constituent of the DNA molecule, is probably a precursor of thymidine (Schneider, 1955) and it is not impossible that, in certain cases, irradiation selectively impairs thymidylic acid synthesis. Two observations favour this hypothesis. In the rat, urinary excretion of deoxycytidine, but not of thymidine, increases 24 hr after irradiation (Parizek *et al.*, 1958). Potter and Buettner-Janusch (1958) injected irradiated rats (800 r) with ^{14}C-

thymidine and determined, at various times, the radioactivity in the thymidine and thymine nucleotides fractions. Until 24 hr after irradiation, thymidine radioactivity increases fourfold, whereas activity in the thymine nucleotides keeps constant or decreases.

In rat liver, 720 r total-body irradiation bring about an increase of nucleotides; the maximum amount is obtained after 24 hr, but 80 per cent of the increase take place during the first hour after irradiation (Cima *et al.*, 1959). Pigeon liver, too, displays a temporarily enhanced hypoxanthine synthesis after 2000 r; this rather high dose produces, thereafter, a drop of synthesis (Kritsky, 1959b). Maas and Schubert (1958) observe a rise of concentration of nucleoside monophosphates in rat thymus after 800 r total-body irradiation.

It is possible that nucleotides might accumulate erratically, after irradiation, without regard to the normal ratios between purine and pyrimidine bases. DNA synthesized from this material, once the inhibition period is overcome, might not be normal. Ascites tumour cells synthesize DNA at a normal rate after 400 r (Kelly, 1957), but have provisionally stopped dividing. When the mitotic activity is resumed, Conger (1956) spotted anaphase anomalies in almost all cells, which may be the consequence of abnormal DNA synthesis.

Other examples of the presence of abnormal DNA after irradiation may be cited. Two days after 400 r total-body irradiation, rat spleen DNA has a higher content of guanine and adenine and a lower content of cytosine and thymine (Berenbom and Peters, 1956). Antigenic properties of rat spleen deoxyribonucleoprotein are altered 24 hr after total-body irradiation (Jankovic *et al.*, 1957). In normal yeast cells, DNA hydrolysis in hot acid yields apurinic acid, whereas in cells irradiated by 2×10^5 r, purine bases still persist beside thymine in DNA after acid hydrolysis. By acidifying an alkaline hydrolysate of DNA (according to the Schmidt–Tannhauser technique), the DNA precipitates in the normal yeast cells, but not in the irradiated cells (Chantrenne and Devreux, 1959).

Errera (1957) and Mandel and Chambron (1959) offer the hypothesis that accumulation of nucleotides and the ulterior synthesis of abnormal DNA might be the cause of the mutations induced by ionizing radiations. This attractive hypothesis requires, however, that the accumulation of nucleotides observed in spleen and thymus be also recovered in the gonads. It seems that all the tissues do not accumulate nucleotides after irradiation. In bone-marrow, for instance, Graevsky (1958) and Kritsky (1959a) find a decrease in content of nucleotides, already marked 5 min after 700 r. That local irradiation of the marrow brings about the same effect proves, according to Graevsky, that the marrow has lost, by irradiation, the ability to stock the purine nucleotides which are

normally supplied by the liver (Lajtha and Vane, 1958)—since the marrow seems unable to synthesize its purine bases either *in vitro* (Davidson *et al.*, 1957) or *in vivo* (Lajtha and Vane).

This lack of purine synthesis by marrow cells *in vitro* may be responsible for the fact that ^{32}P incorporation into DNA is enhanced by addition of deoxyadenylic acid to a marrow suspension drawn after total-body irradiation (Drasil and Soska, 1958).

DNA metabolism does not seem homogeneous. Morin *et al.* (1957) have separated by differential centrifugation ground liver cell nuclei in various sub-fractions, each of which incorporates ^{32}P into DNA at its own rate. The bulk of the DNA of a rat carcinoma may be split into two fractions, only one of which is soluble in 0·9 per cent NaCl: both fractions incorporate ^{32}P at the same rate in normal tumours, but at different rates in irradiated tumours (Harbers and Backmann, 1956). These experiments disclose many still unexplored possibilities.

Before undergoing polymerization, the nucleotides must be phosphorylated to their triphosphates. This stage seems to be affected by X-rays. Creasey and Stocken (1958, 1959) observed that within 1 hr, the formation of high energy phosphorus compounds by nuclear suspension was suppressed by 100 r; it is only 20–50 per cent of normal after 25 r (total-body irradiation). The inhibition can already be detected 3–5 min after 100 r. Only nuclei from radiosensitive tissues (thymus, spleen, lymph nodes, bone-marrow and intestinal mucosa) are capable of phosphorylating nucleotides; liver, kidney, brain and pancreas are not. This function begins to recover from radiation damage in spleen and thymus 3 days after 100 r, but not after 1000 r. In rats injected with cysteamine 5 min before irradiation, nuclear phosphorylation is less inhibited by 100 r (90 per cent instead of 100 per cent after 1 hr), and 90 per cent of the control values are already recovered after 48 hr.

Potter (1959) has recently confirmed these results: within a few hours, from 100 to 3200 rep γ-rays (^{60}Co) inhibit the incorporation of ^{14}C-thymidine into thymine nucleotides and, to a still greater extent, into DNA. In fibroblasts irradiated *in vitro*, however, Paul (personal communication) does not find any drop in ^{3}H-thymidine incorporation into thymidine triphosphate, although incorporation into DNA is depressed. Phosphorylation mechanisms would thus be much less radiosensitive in this type of cells than in the tissues studied by Creasey and Stocken.

Another confirmation is given by Maas and Schubert (1958): in rat thymus, after total-body irradiation, nucleoside monophosphates increase in amount whereas nucleoside di- and triphosphates decrease.

By measuring ^{32}P incorporation rate into rat thymus DNA 2 hr after increasing doses of irradiation, Ord and Stocken (1958a) observe

a rapid increase of inhibition for doses below 200 r (50 per cent for 200 r) and a slower one beyond (75 per cent inhibition after 1600 r). The first more radiosensitive stage, might correspond, according to the authors, to an enzymic inhibition such as that of intranuclear phosphorylation. The second stage might reflect damage to the DNA molecule itself. Lajtha *et al.* (1958) reach a similar conclusion by studying by the autoradiography technique the incorporation of ^{14}C-formate into bone-marrow cells *in vitro* after irradiation with increasing doses.

If the radiolesion is located somewhere before nucleotide triphosphates are formed, then the polymerization mechanisms might have remained normal, but not working for lack of normal precursors. This, however, does not seem to be the case since the recent work of Potter (1959) shows that the polymerization is still more inhibited by irradiation than the formation of thymidine nucleotide. It is therefore likely that the inhibition of DNA synthesis may be ascribed to the inhibition of two enzymes or groups of enzymes: the nucleotide kinases and the DNA polymerase.

All the nucleic acids synthesis performed up to the present time *in vitro* require polymerized DNA or RNA as primer (Grunberg-Manago, 1956, for RNA; Kornberg *et al.*, 1956, for DNA). If the same requirement also applies to *in vivo* conditions, it is conceivable that damage to the primer by X-rays might induce faulty priming of polymerization or no polymerization at all. However, one might object that, according to Wheeler and Okada (1959), previous irradiation of primer DNA by moderate doses (18,000 r γ-rays) leads to an *increase* of *in vitro* synthesis and that higher doses are required to produce a decrease. In the *in vitro* synthetic systems, highly polymerized DNA is a bad primer, as is well known: it must be degraded to some extent in order to prime the synthetic reaction, and this may be what Wheeler and Okada actually do by irradiating it. But if one admits, by sheer analogy, that the phenomena might be the same *in vivo* as *in vitro*, in that case the alteration of DNA molecule in the nucleus would not matter as far as DNA synthesis is concerned.

7. *Conclusions*

In mammals, extended observation of *in vivo* effects of X-irradiation on nucleic acids biosynthesis is confused by two factors: cell death and changes in cell population after irradiation.

Relatively high doses of X-rays inhibit DNA synthesis. Lower doses delay it without inhibiting it very much, provided they are given before the onset of synthesis. A few cases of acceleration of synthesis by very low doses have been described.

X-rays do not impede the synthesis of nucleotides which accumulate

in many irradiated tissues. The inhibition of DNA synthesis may be ascribed to:

(a) A decrease in phosphorylation of nucleotides leading to nucleotide triphosphates.

(b) An inhibition of the specific polymerase.

II. NUCLEASES

NUCLEASE activity is almost always increased in tissues and body fluids of an irradiated mammal. Many publications on that subject have been reviewed by Bacq and Alexander (1955), Holmes (1957), Errera (1957) and Stocken (1959). Most of them deal with DNAases (deoxyribonucleases) and will be considered in the last section.

1. *Ribonucleases*

Rat liver contains an alkaline ribonuclease (RNAase) with an optimum pH 7·8 to 8·2, and an acid RNAase having the optimum pH 5·8. (De Lamirande *et al.*, 1954; Roth, 1954). A protein of the soluble fraction of the cytoplasm is an inhibitor of alkaline RNAase (Roth, 1956). Pirotte and Desreux (1952) had already noticed that rat liver supernatant fraction was rich in alkaline RNAase inhibitor and that treatment with 0·25 N H_2SO_4 could inactivate the inhibitor and release, in all fractions, a high RNAase activity. The intracellular distribution of the two enzymes is somewhat different: acid RNAase is mainly located in lysosomes (De Duve *et al.*, 1955) whereas alkaline RNAase is present as partially active enzyme in the granules and as totally inactivated complex in the supernatant.

In rat liver, after 600 r total-body irradiation, acid RNAase activity increases by 50 per cent from the second day onwards; it drops back to normal at the fifth day and decreases further to reach 25 per cent of control value at the eighth day; the normal activity is restored twelve days after irradiation (Roth *et al.*, 1953). In a more recent work, Roth (1956) confirms his previous observations on acid RNAase. He does not find any modification of alkaline RNAase activity in the supernatant fraction although the inhibitor content is lowered at first, 24 hr after irradiation and comes back to normal at the second day. A decrease in mitochondrial RNAase occurs only five days after irradiation.

In more radiosensitive organs such as spleen and thymus, the variations seem more clear-cut. According to Roth (1958), 700 r total-body irradiation modify the intracellular distribution of rat spleen acid RNAase: mitochondrial and supernatant activities vary from, respectively, 36 and 28 per cent in the controls to 29 and 44 per cent after 16 hr, and 15 and 66 per cent after 24 hr. Since the total activity of

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spleen homogenate acid RNAase is doubled 16 hr after irradiation, the mitochondria certainly contain a higher absolute activity at that moment than the controls, despite the decrease in percentage of total activity. Nevertheless, 64 hr after irradiation, the mitochondria seem to have lost acid RNAase in favour of the supernatant, because total activity is slightly lower than in the controls.

In mouse thymus (Weymouth, 1958), from 40 to 160 r total-body irradiation provoke an enhancement of activity of both RNAases per cell (that is, per mg DNA) which already begins 20 min after irradiation for alkaline RNAase and 8 hr after for the acid enzyme. A maximum is attained 24 hr after irradiation and represents ten times and twice the normal activity for alkaline and acid RNAases, respectively. Alkaline RNAase concentration (per milligram thymus) is five times greater 24 hr after irradiation, whereas acid RNAase concentration hardly changes, contrary to its absolute amount. Between 40 and 160 r, the increase of alkaline RNAase is proportional to the dose, but that of acid RNAase is dependent on time only, not on dose. According to Weymouth, changes in cell population cannot explain the rise in RNAase activities by irradiation. A gradual destruction of inhibitor is a plausible explanation, since the inhibited enzyme, alkaline RNAase, increases with the dose of radiation and the acid, non-inhibited, enzyme does not.

It is difficult to appreciate the incidence of RNAases activation on RNA metabolism. Being hydrolases, they may simply impede the RNA synthesis by shifting the equilibrium towards hydrolysis. But it may also not be so simple. Crystallized RNAase, added to Landschütz tumour cells, first raises the RNA/DNA ratio, then lowers it below normal level. The initial rise corresponds to the synthesis of an abnormal RNA which has more cytosine and less guanine (Ledoux, 1956). In this case, added RNAase doubles the incorporation rate of uracil and cytosine without affecting adenine and guanine incorporation; it also disturbs DNA thymine metabolism (Ledoux and Vanderhaeghe, 1957). Whether endogenous RNAase can have similar actions is not yet known.

Till now, no abnormal RNA has ever been detected after irradiation.

It should also be remembered that RNAase is an inhibitor of growth for onion root cells (Brachet, 1955) and for chicken fibroblasts *in vitro* where it prevents mitosis without affecting DNA synthesis (Firket *et al.*, 1955). All these questions are discussed in the recent textbook by Brachet (1957).

On the other hand, if exogenous RNAase displays an antimitotic action *in vitro*, it is not at all certain that endogenous RNAase would behave in a similar way, since Brody and Thorell (1957) found a *direct* relationship between alkaline RNAase activity and mitotic index in the hen bone-marrow.

2. *Deoxyribonucleases*

Tissues possess a neutral DNAase or DNAase I and an acid DNAase or DNAase II in variable proportions. DNAase II generally predominates within the cells (Allfrey and Mirsky, 1952; Koszalka *et al.*, 1954; Schmidt, 1955; Shack, 1957). Oligonucleotides from DNA hydrolysis by acid DNAase can be further degraded by neutral DNAase (Privat de Garilhe and Laskowski, 1955).

The investigation of DNAases is confused by the presence of inhibitors of neutral DNAase in tissues, blood and urine (Gilbert *et al.*, 1951; Henstell *et al.*, 1952; Kurnick *et al.*, 1953), and of acid DNAase in urine (Kowlessar *et al.*, 1957); an activator has even been detected in the plasma (Feinstein and Green, 1956). Within the cell, acid DNAase is located mainly in lysosomes (De Duve *et al.*, 1955) and neutral DNAase in the mitochondria (Baudhuin, 1959).

2.1. *Quantitative changes.* They are the most evident changes. In rat spleen, acid DNAase activity increases from 6 to 24 hr after 500 r; it drops back to normal at the forty-eighth hour. Meanwhile, the neutral activity, normally rather weak, progressively decreases (Douglass *et al.*, 1954; Fellas *et al.*, 1954). No recovery of normal activity of spleen acid DNAase occurs after 850 r: the activity is increased three-fold 24 hr after irradiation and remains high at later dates (Goutier, 1959). According to Gordon *et al.* (1959), the initial increase of activity may be due to the disappearance of lymphocytic centres, probably poorer in enzyme. Okada *et al.* (1957), however, observed that after irradiation, the DNAase activity increases as much in the connective tissue as in the lymphoid centres of the spleen. Injections of cysteine (Okada *et al.*, 1957) and cysteamine (Goutier, 1959) before irradiation do not modify the initial enhancement of acid DNAase activity.

Very similar results are found by Weymouth (1958) in mouse thymus where acid DNAase activity increases only from the twelfth hour onwards after 160 r. Expressed per cell, the activity is increased six-fold after 24 hr. A higher dose (780 r) raises the activity twenty-fold after 2 days (Kurnick *et al.*, 1959). According to Kurnick *et al.*, acid DNAase increases less rapidly in bone-marrow than in lymphoid organs, but in the former tissue, the activity keeps rising for at least 7 days while it drops after 2 to 3 days in spleen and thymus.

The activation of acid DNAase is smaller in the liver: the activity is normal in rat liver after 500 r (Fellas *et al.*, 1954) and is hardly doubled 4 days after 700 r in mouse liver (Kurnick *et al.*, 1959).

Possible mechanisms for the DNAase activation by irradiation are outlined below:

(a) Fellas *et al.* (1954) suggested that cells of radiosensitive tissues

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might selectively retain acid DNAase while losing many other protoplasmic constituents. Okada *et al.* (1957) have provided an argument in favour of that hypothesis: for 24 hr after irradiation, the specific activity of acid DNAase increases in the spleen, but the total activity remains constant. These authors point out, however, that it is difficult to reconcile this hypothesis with the fact that acid and neutral DNAases increase in blood and urine. Eighteen hours after irradiation, serum acid and neutral DNAase activities are, respectively, twelve times and twice as high as normally (Kowlessar *et al.*, 1955). Urinary excretion of both DNAases is three times greater after irradiation (Kowlessar *et al.*, 1954). Jovanovic and Voncina (1959) have recently found that splenectomy of the rat does not influence the increases of serum and urine DNAases after irradiation: consequently, the enzyme activities increasing by irradiation in these two fluids do not originate from the spleen. Moreover, splenectomy by itself, without ulterior irradiation, raises the DNAase activities in serum, urine and bone-marrow to the same extent as do X-rays. The authors conclude that irradiation performs a sort of functional splenectomy and that DNAase activation in blood, urine and bone-marrow might be ascribed to the loss of a metabolic regulation from the spleen.

In these conditions, the hypothesis of a retention of DNAase might be detained for the spleen.

(b) Modifications of cell population after irradiation cannot explain the increase of DNAase activity in the thymus (Weymouth, 1958) nor in the spleen, since connective tissue and lymphoid follicles display similar increases in DNAase activity (Okada *et al.*, 1957).

(c) Activation of DNAases might also result from alterations of inhibitors and activators present in tissues. Recently, Kurnick *et al.* (1959) observed a marked decrease of the inhibitor of serum neutral DNAase in the spleen, from 1 to 2 days after irradiation. Yet, neutral activity remains very low in spleen, even after irradiation. Since no inhibitor of acid DNAase has ever been found in tissues, it is hard to admit, even by analogy with neutral DNAase, that irradiation activates acid DNAase by destroying an inhibitor.

(d) Within the cells or in a homogenate containing intact granules, a great proportion of acid DNAase is enclosed in the granules and cut off from its substrate. One of the activation mechanisms might be the release of DNAase from its bound state. Bacq and Errera (1956) have already postulated that release of intragranular enzymes, especially of hydrolases, might be one of the main lesions of ionizing radiations. Their views are confirmed by the observation that RNAase (Roth, 1958) and acid DNAase (Goutier-Pirotte and Thonnard, 1956) increase in spleen supernatant after irradiation. But more thorough investigations

are lacking on intracellular distribution of DNAases after irradiation.

There is no doubt that irradiation damages the intracellular granules (McQuade *et al.*, 1956; Ryser *et al.*, 1954). Even *in vitro*, mitochondria respond to small doses of radiation: Fritz-Niggli (1956a, b) observed an inhibition of the oxydation of citrate by a suspension of mitochondria and a greater fragility of the granules after irradiation with 50 r.

By homogenizing spleen tissue in 0·44 M sucrose, the integrity of the granules is preserved and the acid DNAase activity measured is one-third of that in water homogenates where the granules are disrupted. With an irradiated spleen, the difference between the activities in water and sucrose homogenates is less pronounced (Okada *et al.*, 1958). Therefore, irradiation allows access of the substrate to a greater proportion of acid DNAase in sucrose homogenate. But Okada *et al.* also observed that the activity in water homogenates increased after irradiation *in vivo:* since it represented the total activity in the controls (proved by the absence of further activation by supersonic vibrations) one must admit that DNAase has been not only activated, but also synthesized. In suspensions of liver mitochondria irradiated *in vitro* by γ-rays, Okada and Peachey (1957) observed a rise of acid DNAase activity, but the enzyme remained bound to the granules although the mitochondria were broken up when looked at in the electron microscope. In this case, the disruption of the granules simply allowed enzyme and substrate to come into closer contact without actual release of the enzyme. DNAase would then, according to Okada and Peachey, be bound to the outer and inner mitochondrial membranes. Of course, this concerns only liver mitochondria *in vitro*. One can easily visualize how the simple disruption of the granules without release of the enzyme activates the DNAase *in vitro*, because the substrate is present in the medium in relatively high concentration. But in a living cell, the substrate is in the nucleus and the simple disruption of the mitochondria (or lysosomes) will not bring enzyme and substrate close together unless DNAase diffuses out of the granules.

As for RNAase, it is still difficult to understand the full meaning of the activation of DNAases after irradiation, because the physiological role of acid DNAase remains obscure. Recent works suggest that DNAase might have another role than a purely hydrolytic one. Brody and Thorell (1957) and Brody and Balis (1959) found an increase of acid DNAase activity in growing tissues. Goutier *et al.* (1959) observed a similar increase during the first hours of regeneration in rat liver. Chevremont *et al.* (1957, 1959a, b) induced the synthesis of a Feulgen-positive and ^{3}H-thymidine incorporating material in mitochondria by treating fibroblasts *in vitro* with purified acid DNAase. A synthetic role of DNAase is therefore not definitely precluded.

It must be finally emphasized that activation of DNAases is not an early phenomenon: it occurs rather late after irradiation (several hours in some cases), a relatively long time after important lesions of nucleotide phosphorylation and DNA synthesis have already been detected.

2.2. *Qualitative changes.* Very few are known. We described modifications of the pH-activity relationship of acid DNAase in rat spleen, within 30 min after 850 r total-body irradiation (Goutier *et al.*, 1957, 1959). Besides the normal optimum pH 5·15 there appears a second peak at pH 4·8. Since it is possible to obtain similar alterations in the course of the chemical purification of the enzyme, and to get an enzyme with one optimum pH 4·8 after more complete purification, we suggested the hypothesis that spleen acid DNAase would normally be combined with an unknown substance, the complex having the normal optimum pH 5·15. X-rays and chemical manipulations (purification) would split the complex and the optimum pH of the released enzyme would then become 4·8. These qualitative modifications produced by irradiation precede any increase in total activity; they are not specific effects of ionizing radiations.

3. *Conclusions*

Total-body irradiation causes an increase of nuclease activities in radiosensitive tissues. The activation of enzymes may be ascribed to:

(a) Selective retention of the enzyme (possible for the spleen only).

(b) Decrease in tissue inhibitors, proved for alkaline RNAase and neutral DNAase, but not for the acid enzymes which, however, are often more highly activated.

(c) Easier access to substrate by disruption of the granules and eventual release of enzyme in the soluble cytoplasm.

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THE EFFECT OF IONIZING RADIATIONS AND TUMOUR-CHEMOTHERAPEUTIC AGENTS ON THE BONE MARROW

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THE EFFECT OF IONIZING RADIATIONS AND TUMOUR-CHEMOTHERAPEUTIC AGENTS ON THE BONE MARROW

L. G. Lajtha

I. Introduction

It has been known, since the pioneer work of Heineke over 50 years ago (Heineke, 1903) that haemopoietic tissues are among the most radiosensitive, in some species the most radiosensitive, in the mammalian organism. Most of the early work was based on changes produced in the peripheral blood, and on bone marrow histology. Considering that the first is only an incomplete reflection of bone marrow activity (extramedullary stores of cells or changes in peripheral destruction rate may also influence it), and that the second is only crudely quantitative, it is remarkable how well the more recent quantitative methods confirm the early observations.

Previous reviews (Jacobson, 1954) dealt mainly with the "haematological effects" of ionizing radiations, and not primarily with direct effects on the bone marrow. This survey will attempt to deal primarily with direct bone marrow effects, considering peripheral blood changes only where they can be quantitatively related to bone marrow changes.

It is felt that before proceeding to describe and then to attempt to explain the effects of various agents on the bone marrow a brief explanation of the rather special composition and kinetics of this organ is necessary.

II. Composition and Kinetics of the Bone Marrow

Bone marrow is a very heterogeneous population of differentiating cells. Fig. 1 shows the main cell lines: the normoblastic series engaged in red cell production, the myelocytic series in granulocyte (neutrophilic, eosinophilic and basophilic) production, megakaryocytes forming platelets, and lymphatic foci forming lymphocytes. The affiliations of two other series, monocytes and plasmacytes, are not well understood: the former are thought by some haematologists to originate from the reticulum as a separate line of cells, but others feel that they

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Blast Cells
Pronormoblasts
Basophilic normobl.
Polychromatic normobl.
Reticulocytes
Promyelocytes
Myelocytes
Metamyelocytes
Band cells
Polymorphs
Promonocytes
Monocytes
Megakaryocytes
Platelets
Prolymphocytes
Lymphocytes

FIG. 1.

are functional forms of cells which may originate from young myeloid or lymphoid elements; the plasmacytes are considered by most workers to be certain functional forms of lymphoid elements.

It is thought that all "pro-" forms originate from blast cells—it is still a subject of debate whether from different blast cells or from one multipotential blast cell—and that blast cells originate from reticulum cells. Mere morphological criteria, however, are not the happiest of research tools, and the division between reticulum cells and blast cells and blast cells and "pro-" cells is not unanimously agreed upon.

From the "pro" cells onwards the morphological criteria are clear but in every normal marrow there will be at least 10–15 per cent "unrecognized" cells which will remain unclassifiable. The "pro" cells differentiate and divide, but after a certain degree of differentiation has been reached (late polychromatic normoblast, late metamyelocyte) the cells will be incapable of further division and will mature only. As there is evidence that maturation proceeds at a similar rate in cells of similar type, this implies that the cells have a finite life span in the marrow after which they enter the peripheral blood.

It is particularly important to realize that most of the marrow cells are not a self-maintaining population—they have an intrinsic finite life span as illustrated in Fig. 2. It is, however, postulated that there is a

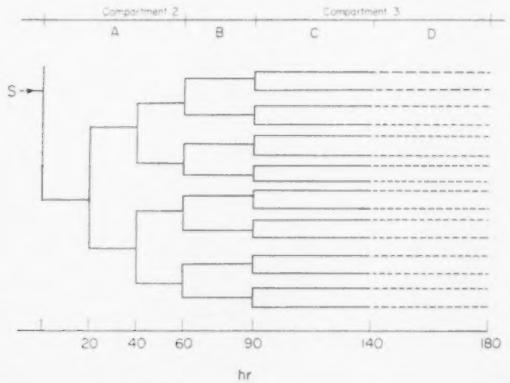

FIG. 2. Simplified scheme of a bone marrow cell population. S = stem cells (compartment 1); A and B dividing and differentiating cells (compartment 2); C and D non-dividing differentiating cells, e.g. late polychromatic normoblasts and reticulocytes (compartment 3).

self-maintaining stem cell population from which cells are "fed" into the differentiating compartment, thus maintaining a steady state equilibrium. It is not the purpose of this article to attempt to identify the stem cells, or to argue whether they are multipotential or not—it is sufficient to accept a self-maintaining stem cell compartment which

regulates its own size, and from which a certain number of cells are induced by certain stimuli to start the process of differentiation. During the course of this process the cells may divide several times, thus a single stem cell starting to differentiate gives rise to a number of differentiated end cells (erythrocytes, polymorphs). The kinetics of this process will be discussed in detail elsewhere (Lajtha and Oliver, 1960a.)

The bone marrow cell population therefore can be divided into three groups or "compartments": (1) Stem cells (probably less than 5 per cent of the total). (2) Differentiating and dividing cells (about 30–40 per cent of the total). (3) Non-dividing differentiating and end, i.e. mature, cells (about 60 per cent of the total). Most of the experimental work reported on the effects of radiations or tumour chemotherapeutic agents on the bone marrow is concerned with the second group of cells. It will be pointed out, however, that the reactions of this second group of cells are only of secondary importance, in spite of the very marked changes which may be induced in them by radiations or similar agents.

III. Effects of Ionizing Radiations on Mammalian Cells

The effects of ionizing radiations on cells may be classified under three main headings (1) Mutagenic effects. (2) Effects on reproductive integrity (Gray, 1959). (3) Effects on enzymes other than those involved in reproductive integrity. The mutagenic effects are outside the scope of this survey, and as the effects listed under (3) require doses about 100–1000 times higher than those affecting reproductive integrity, it is the latter that will be discussed here.

Damage to the reproductive integrity of cells may demonstrate itself in the following ways

(a) Temporary delay in entering mitosis.
(b) Permanent inhibition of mitosis and giant cell formation.
(c) Death of cell before entering mitosis (interphase death).
(d) Death of cell(s) after a few (from one to five) mitoses.

The mechanism by which mitotic delay is produced by radiation is not understood. It appears that (at least in plant cells) the whole of the interphase is "sensitive" in respect of this delay (Neary *et al.*, 1959). In bone marrow cells a delay in the period preceding DNA synthesis has been observed following 150–300 rads, a dose which does not appear to affect the actual process of DNA synthesis (Lajtha *et al.*, 1958a). However, it is not known whether the mitotic delay is due to a complexity of processes or to one key process (Creasey and Stocken, 1958).

Gray, in his analysis of radiation effect on the reproductive integrity of cells (Gray, 1959) groups (b) (c) and (d) together, and makes the important general statement, that, following radiation injury, "the

number of cells in the process of degeneration at any one time will necessarily be proportional to the product of the rate at which cells are entering the degenerative phase, and the length of time they occupy in that phase." The term "degeneration", in this context, embraces such effects as death of cells not normally dividing (e.g. small lymphocytes), and death of cells after from three to five mitoses. Although, until such time that the mechanism of radiation-induced cell death is properly understood, attempts to differentiate between apparently different modes of cell death are of academic importance, the problem is sufficiently basic to be of very great interest.

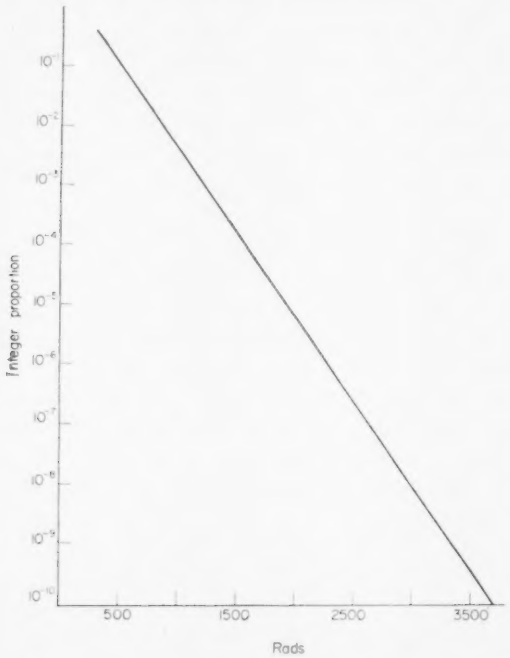

FIG. 3. X-ray dose–response curve for mammalian cells. (After data of Puck and Marcus 1956, and Hewitt and Wilson, 1959.)

Furthermore, the experiments of Puck (Puck and Marcus, 1956; Puck, 1959) and Hewitt (Hewitt and Wilson, 1959), taken in conjunction with observations on the degree of chromosome damage (proportion of damaged mitoses) (Doniach and Logothetopulos, 1955; Holmes and Mee, 1955; Gray and Scholes, 1951; Bender, 1957) indicate the remarkable fact that a certain dose of radiation affects the reproductive integrity of a very similar proportion of cells, irrespective whether they are plant cells or mammalian cells, normal cells or tumour cells, cells

in vitro or cells *in vivo*, irrespective of what organ they originate from.

The conclusion, which may be drawn (although many more experimental observations are required before accepting it as a general law) is, that, at present, there seems to be no evidence for a significant variation in radiosensitivity, in terms of reproductive integrity, between different types of mammalian or plant *cells* (micro-organisms excluded).* The dose–response curves are exponential and the one illustrated (Fig. 3) may in fact be the average curve for mammalian cells. The initial difference in radiosensitivity as observed in different *organs* then is due to the rate at which different types of cells may enter the degenerative phase after irradiation.

IV. PATTERNS OF RESPONSE OF CELL POPULATIONS TO IRRADIATION

1. *Exponentially growing populations*

In an ideal state of logarithmic growth, every cell of the population divides at regular intervals, i.e. the population number doubles at the end of each average intermitotic cycle. Doses of radiations which produce (a) only a delay in mitosis, or (b) impairment of reproductive integrity will therefore produce the growth patterns illustrated in Fig. 4. The steepness of the fall in numbers (Fig. 4b) will depend on the

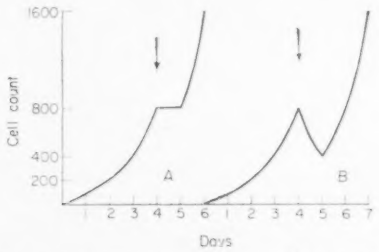

FIG. 4. Response of an exponentially growing population of cells to irradiation. *A* = result of mitotic delay only; *B* = result of 50 per cent cell death. Arrow indicates time of irradiation.

rate at which cells enter their degenerative phase after radiation injury —this may be correlated in many instances with the rate at which cells enter mitosis, i.e. growth rate of the population. Recommencement of growth may modify the descending parts of the curve.

There are only few temporary occasions in a healthy mammalian organism when logarithmic growth occurs (early embryonic growth, tissue repair).

* However, Puck indicates (Puck, 1959) that the observed differences in radiosensitivity (in terms of reproductive integrity) between mammalian cells, and micro-organisms may be due to differences in their DNA content.

Populations which may show logarithmic growth are long-term tissue cultures (both normal and malignant), and tumour growth *in vivo*. It should be remembered, however, that most tumours are not ideal exponential populations: a considerable proportion of their cells may die of "accidents", or lie dormant due to metabolic irregularities (either intrinsic or extrinsic, e.g. insufficient vascularization of the tumour). Hence, determination of the rate of tumour growth (size or cell numbers) does not give a true measure of the rate of multiplication of its cells.

2. *Steady-state populations*

These are characterized by the fact that their cells have a mean life span (either finite, due to differentiation, or average, due to random destruction). These are the populations normally found in healthy mammalian organisms. Their steady state is controlled by some homoeostatic mechanism which enables them to increase or decrease cell production on demand. Bone marrow is a good example of a steady-state population of cells: the stem cells (group 1) randomly differentiate into the "differentiating and dividing" compartment (group 2) while maintaining their numbers by a steady rate of division, and the group 2 cells, after undergoing from three to five divisions (differentiating all the time) eventually enter group 3, the non-dividing differentiated "end" cells.*

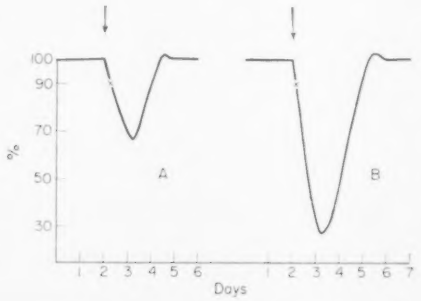

FIG. 5. Response of a steady-state population of cells to irradiation. *A* = result of mitotic delay only; *B* = result of 50 per cent cell death. Arrow indicates time of irradiation.

As such cells have a steady rate of destruction, even a temporary hold-up in mitosis (production) will cause a decrease in their numbers (Fig. 5a). Doses of radiations impairing reproductive integrity will

* The above concept of bone marrow regulation is somewhat different from that proposed by Osgood (summarized, Osgood, 1959), in so far as it eliminates the necessity for the "alpha, *n*" type of division.

cause an even greater drop in numbers (Fig. 5b). In practice the two effects will not be separable.

Again, the steepness of the drop in numbers will depend on the rate at which cells will enter their degenerative phase, but in the hypothetical case of a mere temporary hold-up in production the steepness will depend on the rate of normal destruction (which in steady state equals the rate of normal production). It may be expected, therefore, that in a fast-growing cell population (or in which cells enter their degenerative phase at a fast rate) the drop will be steeper than in a slow-growing population (or in which cells enter the degenerative phase slowly). With the drop in numbers the homoeostatic mechanisms come into function, effecting regeneration. However, effective regeneration may not be able to keep up with a really steep drop in numbers, which—following certain doses of radiation—may reach "danger level". The scheme of events is illustrated in Fig. 6.

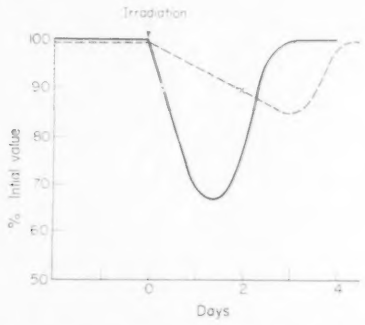

FIG. 6. Response of two steady-state population of cells to the same dose of radiation. Single line = cells with fast rate of degeneration; interrupted line = cells with slow rate of degeneration. X marks the degree of depopulation at which regenerative processes (homoeostasis) may start.

V. EXPECTED RADIATION EFFECTS ON BONE MARROW

FROM the foregoing one may draw up the theoretically expected effect of a single dose of radiation (e.g. 200–300 rads) on the bone marrow (see also Fig. 7).

(*a*) The immediate mitotic delay following irradiation will start an immediate decrease in numbers of the second group (differentiating and dividing) of marrow cells as their rate of removal (i.e. entry into group 3) remains unchanged. Later, when group 2 has been depleted, the "feed" into group 3 will be decreased, and as the rate of removal (into the peripheral blood) remains unchanged the number of cells in group 3 will decrease also.

(*b*) In the meantime stem cells (group 1) will continue to differentiate into group 2, but, as they also have received the same radiation damage, the subsequent mitoses will be delayed and interphase death may also take a toll of the group 2 cells thus produced.

FIG. 7. Scheme of the theoretically expected effect of a single dose of radiation (e.g. 200–300 rads) on a group 2 bone marrow cell population. *A* = initial drop: *B* = abortive recovery; *C* = secondary drop; *D* = final recovery with overshoot.

(*c*) After cessation of the delay (cell-cycle delay, mitotic delay) an abortive "recovery" may follow, i.e. cells may enter into mitosis, but, depending on the dose of radiation received, a large proportion of them will have lost their reproductive integrity and will undergo only one or two mitoses—resulting in a suboptimal temporary rise in their numbers.

(*d*) After a time lag the integer portion of stem cells—regulated by the internal homoeostatic mechanism of the stem-cell group—will regenerate (delayed by the continued drain on their numbers by differentiation into group 2 cells) and increase the size of the stem population to (or perhaps even above) normal. Thus group 2 also will return to normal population size, very likely with a slight "overshoot" owing to increased demand (increased stimulation for differentiation into group 2).

Gray has suggested (Gray and Scholes, 1951) that, as degeneration following irradiation takes a certain time to develop, with increasing time after irradiation the apparent damage may increase. Thus the "lag" phase before regeneration may be considerable. With larger doses of radiations (400–600 rads) the lag phase may be long enough to result in a peripheral blood situation incompatible with life of the organism (chance given for fatal haemorrhage of septicaemia).

It is perhaps permissible to theoretize if one can support theories

with observations. In the following section a brief survey will be made of the observed radiation effects on bone marrow, and an attempt to correlate them with the above scheme of events.

VI. OBSERVED RADIATION EFFECTS IN BONE MARROW

1. *Effects on mitosis*

The first to follow the quantitative changes in bone marrow cells after irradiation was Osgood (Osgood and Bracher, 1939; Osgood, 1942) who attributed the decrease in numbers in his cell suspension cultures to inhibition of cell proliferation. Osgood's findings and conclusions were amply corroborated by subsequent investigators (Rachmilewitz *et al.*, 1945; Gunz, 1949; Boll *et al.*, 1956). The quantitative aspect of radiation damage to bone marrow mitoses was found to be similar *in vivo* and *in vitro*, i.e. about 65 per cent abnormal anaphases after a dose of 200 r *in vivo* in mice (Devik, 1952), and 50–75 per cent depression of the mitotic index following colchicine treatment *in vitro* after 50–500 r (Astaldi, *et al.*, 1950).

An "indirect" effect on bone marrow cell mitosis was observed by Gunz (1953), who noted a temporary inhibition of mitosis in the bone marrow 80 min after irradiation of the spleen in six out of twelve patients with chronic myelocytic leukaemia. Under his conditions the sternal marrow received between 1·5–4·5 r, a dose in itself insufficient to produce the observed effect. The clinical response of patients with chronic myelocytic leukaemia to localized splenic radiation is well known although its mechanism is not understood. Further work will have to establish whether the effect observed by Gunz is specific for this type of leukaemia and/or spleen.

A slowly dying belief amongst some radiotherapists is the "stimulating" dose of radiation. This is partly based on the slight increase of mitotic index in some tissues, including bone marrow, following 5–10 r (Martin *et al.*, 1955). It is emphasized, however, that there is no good evidence that anything but degenerative changes follow irradiation of cells. These changes may initiate, after a time, a compensating temporary hyperactivity, but, even so, many of the ensuing mitoses will be damaged. Indeed, a certain type of chromosome damage may delay the actual process of mitosis—an elongation of the mitotic process resulting in an increase of the mitotic index has been described in marrow erythroblasts (Boll *et al.*, 1956)—but it would be seriously misleading to describe it as "stimulation".

2. *Population changes in the bone marrow*

In a careful serial study of bone marrow histology following various doses of total body irradiation of rabbits, guinea pigs, rats and rabbits

Bloom noted that as early as ½ hr after 800 r there was evidence of damage in the bone marrow, first indicated by cessation of mitotic activity and by a reduction in numbers of erythroid cells (Bloom, 1948).

Subsequent quantitative work has confirmed Bloom's observation, and it is generally agreed that the erythroid and lymphoid population of the bone marrow is depleted faster than the myeloid series of cells (Rosenthal *et al.*, 1951; P. F. Harris, 1956; Hulse, 1957, 1959a, b). The megakaryocytes seem to parallel the myeloid cells (Lindell and Zajicek, 1957).

In general, the pattern of response to moderate doses of radiations (100–400 rads) follows that already illustrated in Fig. 7. An initial decrease in numbers is followed by an abortive recovery, then a further decrease in numbers and finally a true recovery, usually with an "overshoot". In the erythroid and lymphoid series the initial drop reaches maximum by about 24 hr in the myeloid series at about 5 days.

The rate of "true" recovery appears to be more dose dependent than the initial drop in numbers. This is perhaps understandable if it is accepted that a mere temporary mitotic delay is sufficient to depopulate temporarily the group 2 cells (see previous section), and that the proportion of stem cells damaged, and thus the rate of recovery, will be related to the dose (Fig. 3).

It is also generally agreed, that the "early" marrow cells, i.e. pronormoblasts, basophilic normoblasts, promyelocytes, early myelocytes (group 2 cells) are depleted first, followed by the "late" cells, i.e. polychromatic normoblasts, metamyelocytes (group 3 cells). In fact, the rate of depletion of early normoblasts and promyelocytes is very similar—the difference between the erythroid series *in toto* and of the myeloid series *in toto* is due to the slower rate of depletion (after an initial rise) of the late myelocytes, metamyelocytes and band cells.*

To determine the rate of depletion of the different cell types close attention has to be paid to the time sequence of events, it is not sufficient to take a single point, e.g. 24 hr after irradiation, since that time may coincide with the abortive recovery peak of the early normoblasts and the maximum initial depletion of the late normoblasts. It was this coincidence of two different events that led Hulse to conclude that the late normoblasts are more radiosensitive than the early normoblasts, although the initial fall in numbers was significantly sharper in the early normoblasts (Hulse, 1957), an observation also reported by E. B. Harris (1958).

* If the marrow reticulocytes would also be included, the rate of the depopulation of the erythroid series *in toto* may not be significantly greater than that of the myeloid series of cells.

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Most of these quantitative investigations (which involve a great deal of careful work) were performed on rats (Hulse, 1957, 1959a, b; E. B. Harris, 1958) or guinea pig (P. F. Harris, 1956). While for technical reasons these small rodents are advantageous, it should be remembered that the kinetics of erythropoiesis is different in rat and man (in the rat the late normoblasts take up as much ^{59}Fe as the early normoblasts, in man only about half of that of the early cells), and that the lymphatic tissue is more preponderant in the guinea-pig marrow than in human marrow. Nevertheless, qualitative work in rabbits and dogs, animals with marrow kinetics more similar to man, (unpublished observations from this laboratory and of Dr. E. L. Alpen of San Francisco) seems to confirm the above pattern of events, even though the time scale may be slightly longer.

All the experimental evidence cited above agrees well with the concept outlined in the previous sections. The early bone marrow cells (pronormoblasts, basophilic normoblasts, promyelocytes) disappear first, partly because their numbers depend on frequent divisions, and partly because radiations will not prevent their maturation (Patt, 1960). The late normoblasts, myelocytes and megakaryocytes spend a considerable time in maturation, division is less frequent in these cells than in the early ones (the intermitotic cycle appears to be longer in the later forms (Lajtha, 1959))—their rate of degeneration does not appear to be much faster than their normal rate of leaving the marrow. The small lymphocytes appear to be different since these cells enter their degenerative stage following irradiation rather fast. Consequently their rate of disappearance will be similar to the early marrow cells, even though the biochemical mechanism leading to cell destruction may be different. It should be remembered that lymphocytes (and this is a mere morphological term) in different parts of the body do respond differently to radiation, from which Trowell concluded that their response may be considerably influenced by their age or environment or both (Trowell, 1952). It is quite conceivable that the processes of degeneration following irradiation may be influenced (enhanced or delayed) by the milieu in which the cells exist.

3. *Nucleic acid metabolism*

Published work on nucleic acid metabolism of bone marrow following radiations can be divided into two main groups (*a*) in which the nucleic acid metabolism of the marrow *cells* has been investigated, and (*b*) in which the nucleic acid content or specific radioactivity was used to indicate *population changes*. The subject is reviewed in detail elsewhere (Lajtha, 1960) and it may be sufficient to cite the early observation that within 6 hr after 300 r there is already a detectable

change in the marrow nucleic acid content, the latter decreasing to less than half in 2 days (Lutwak-Mann, 1952). This observation, and similar ones made later confirm the population changes described by direct methods.

Radiation effects on the nucleic acid metabolism of the marrow cells are basically similar to those observed in other mammalian or plant cells (Lajtha *et al.*, 1958a). The only difference so far found is that the actual process of DNA synthesis follows a two-component, dose-response curve (Lajtha *et al.*, 1958b) so far only found in thymocytes *in vivo* (Ord and Stocken, 1958).

In any case, it must be remembered that doses of radiation which measurably affect the process of DNA synthesis in mammalian cells are significantly higher than those required to cause profound population changes by affecting the reproductive integrity of cells.

4. ^{59}Fe *studies*

Hennessy and Huff (1950) reported that as little as 5–10 r total-body X-irradiation depresses the amount and delays the appearance of a tracer dose of ^{59}Fe in the peripheral erythrocytes. Since the process of ^{59}Fe incorporation into bone marrow cells is not affected by doses of up to 5000 rads (Lajtha and Suit, 1955; Suit *et al.*, 1957a) it is clear that the post-irradiation ^{59}Fe appearance curve measures erythroid cellularity of the marrow at the time of injection of the tracer. This is also demonstrated by the observation that a certain time has to elapse between irradiation with small doses and administration of the tracer iron (i.e. time for depopulation of the erythroid elements of the marrow) for a significant alteration of the appearance curve. With larger doses (100–300 r) the depopulation may be fast enough to show an effect even if the tracer is administered immediately after (or even prior to) irradiation (Hennessy and Huff, 1950; Belcher *et al.*, 1954; Griffin and Alpen, 1955; Suit *et al.*, 1957b). If radio-iron is given prior to maximum depopulation, following large doses of radiation (600 r) the label from the destroyed cells may be concentrated in iron stores, thus giving relatively high "bone marrow radioactivity" (de Schryver *et al.*, 1957).

The ^{59}Fe studies do not give a very good indication of the intricate population shifts which occur with time after irradiation (proportion of different types of early and late forms of cells), and their interpretation is somewhat complicated by the concept introduced by Lamerton *et al.* (1956), namely that radiation may produce a hold-up in the *release* of existing mature cell types from the marrow. Nevertheless, their performance is considerably easier than the quantitative population analyses mentioned earlier, and, in terms of measurement of the *overall* erythroid activity of the marrow, they are more sensitive. A good

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illustration of the use of the ^{59}Fe method is given by the elegant studies of the role of spleen and limb shielding in the bone marrow recovery after irradiation (Belcher *et al.*, 1958).

The fact that the time for maximum marrow depopulation is the same in the rat whether it is measured by direct cell counts or with the ^{59}Fe method, makes the use of the ^{59}Fe method acceptable in cases where absolute cell counts are impossible, i.e. man. It was found that after a total-body dose of 100 rads the time for maximum initial depopulation was between 48 and 72 hr (Suit *et al.*, 1957b) compared with 24 hr in the rat. In man some recovery was noted by 96 hr. If extrapolation from the rat data is legitimate then the maximum initial depopulation of the myeloid series *in toto* (not the promyelocytes only) after 100 rads would be expected around the eighteenth to nineteenth day.

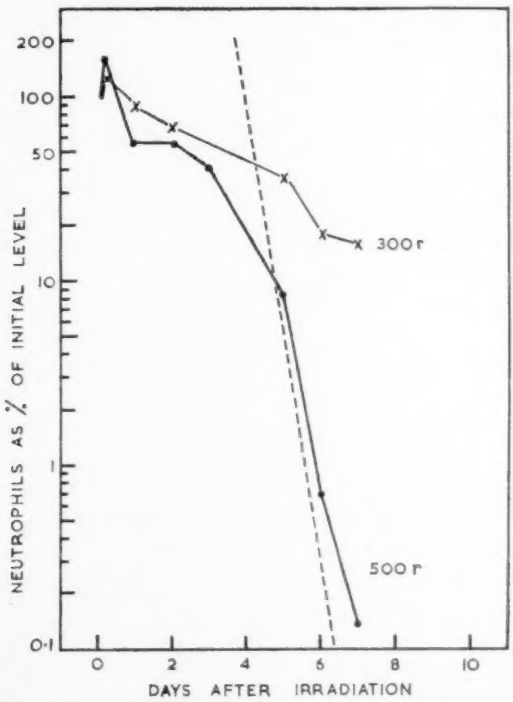

FIG. 8. Fall in blood neutrophil count of dogs after total body irradiation. Interrupted line: normal removal rate of blood neutrophils. Rate of fall after the sixth day following 500 r indicates insignificant (if any) production of new neutrophils. (After data of Patt, 1960.)

However indicative the initial depopulation may be, it should be remembered that after the abortive recovery a second fall will follow.

In fact, the maximum depression of peripheral cells after 100–175 r total-body radiation in man occurs at about 4–6 weeks (Miller *et al.*, 1958; Cronkite *et al.*, 1955). All these time scales would be expected to shorten after larger single doses of radiation.

Although the initial decrease in marrow cellularity is considerable, even with small doses of radiation, some blood-cell production will still continue. This is illustrated in Fig. 8, where the fall in blood neutrophil count after a total-body irradiation with 300 r and 500 r is charted (dogs). The rate of fall after 500 r, after the sixth post-irradiation day is parallel with the normal removal rate of neutrophils (interrupted line), signifying that few, if any, new neutrophils are produced after the sixth day. The rate of fall after 300 r is slower, indicating some granulocyte production still going on. It is noteworthy that, even after 500 r, first some "reserves" have to be exhausted before the steep fall can commence.

5. *Recovery after irradiation*

Bloom noted (Bloom, 1948) that the erythroid series of cells are the first to show depletion, and the first to recover after irradiation. This appears to be confirmed by Hulse's data (Hulse, 1957, 1959a, b). While this appears to be so in the rabbit and the rat, in the guinea pig P. F. Harris (1956) found an earlier recovery of the marrow lymphocytes than of the erythroid cells. This "recovery", however, was temporary, followed by a drop after the sixteenth day, reaching a low point at about 20 days (by which time the erythroid cells have been at their normal level for 4 days, and the myeloid cells have reached normal level and are in the process of "overshooting").

Although recovery can be apparently complete or near complete after repeated doses of radiations (Valentine *et al.*, 1952a Baum *et al.*, 1955), careful analysis of the rate of regeneration may indicate a residual injury, as was found in rats using the ^{59}Fe method (Alpen and Baum, 1959). This residual injury appeared to be accumulating with repeated exposures to radiation, the nature of accumulation suggesting a "decaying exponential relationship".

6. *Effects of continuous irradiation*

The data so far mentioned refer to single or infrequently repeated doses of radiations. With continuous exposure the picture changes considerably. The essential difference between single doses (100–500 rads) and continuous doses (100–2000 rads) lies in the dose rate: relatively high with the single doses employed (10–100 r/min), and low (10–80 r/day) with the continuous doses (this usually implies 16–20 hr radiation per day).

The principal characteristic of response to continuous low dose rate irradiations is that the bone marrow tolerates markedly higher total doses than with the high dose rates (Lorenz *et al.*, 1946, 1947; Henshaw *et al.*, 1947; Evans, 1948; Jacobson, 1954). In the early experiments of Lorenz, the total accumulated dose in mice reached over 5000 r and in rabbits over 10,000 r. A recent serial bone marrow study of guinea pigs (Edmondson, 1958) receiving γ-radiation at the rate of 10 r/day indicated no gross marrow changes until immediately prior to death (total dose of 1140–3450 r). Although myelopoiesis indicated early impairment, the red cell count did not start to drop until a marked fall in the platelet count became apparent (at the level of about 900 r). As even prior to death the marrow indicated active erythropoiesis, and as the cause of death was usually multiple haemorrhages, it may be supposed that red cell production was not significantly impaired (also shown by high reticulocyte counts). Similar studies (Lamerton, *et al.*, 1960), using rats and employing several levels of dose rates confirm the basic findings: large total doses may be accumulated before impairing marrow activity, and when impairment is shown it appears earlier in the myeloid, lymphoid and megakaryocytic series of cells than in the erythroid cells.

There are two obvious questions, raised by the results of continuous low dose rate irradiation: (1) how can cells continue to proliferate after an accumulated dose of 500–2000 r; and (2) what is the explanation of the different behaviour of the erythroid cells following single doses from that during continuous irradiation? (they are the first to show signs of depletion after single doses, and they are the last to be affected—if at all—during continuous treatment).

As to the first question, it is known that radiation delivered at a low dose rate is generally less efficient in producing biological damage, than at a high dose rate. This is illustrated by the results of Baum and Kimeldorf (1957) who found that recovery of bone marrow erythroid activity (^{59}Fe method) after a dose of 240 r to rats was fastest in the group of animals which received the radiation at the rate of 10 r/hr and slowest in the 120 r/hr group.

Quastler *et al.* (1959) have suggested that during long-term continuous irradiation polyploidy may develop in a number of cells, and there is some indication that polyploid cells (because of their "extra" chromosomes) may tolerate larger doses of radiations than diploid cells. While polyploidy may develop under such conditions—the matter is being investigated (Quastler *et al.*, 1959)—there is an alternative hypothesis which would explain the short-term, dose-rate effects as well as the long-term effects (polyploidy could only account for the long-term effects). This hypothesis would consider, that, to affect reproductive

integrity a "multiple injury" type damage is required. If each single injury may "recover" in time, and the simultaneous existence of several of them are necessary for the damage (e.g. Lea, 1946), then, for a certain dose, a certain dose rate will be required to produce the damage in a certain proportion of a cell population. Gray and Scholes (1951) have, in fact, shown that in bean root cells the 37 per cent dose (mean lethal dose) for X-rays varies with dose rate. Also, the X-ray survival curve of mammalian cells (Puck and Marcus, 1956) suggests a "2 hit" type of mechanism. It appears, therefore, that a certain concentration of reversible injuries is required to produce the irreversible damage to reproductive integrity. The equilibrium concentration would depend only on dose rate, but if the life span of the injuries is comparable with the treatment time, equilibrium conditions will not be reached, and the concentration will depend also on total dose.

As to the second question, the following explanation is offered: as radiation effects on the bone marrow ultimately depend on the integrity of the stem cell population, the erythroid cell production is just as much affected initially as the myeloid cell production.

As illustrated in Fig. 2, the early erythroid cells (group 2) undergo five divisions before entering into group 3, and then will spend about 50 hr in group 3 before becoming reticulocytes. If the average early myeloid cell has to undergo more than five divisions, or if in some stages the intermitotic cycle time of the myeloid cell is longer than of the erythroid cells (as has been suggested (Lajtha, 1957)), it would take a longer time for them to reach normal levels. However, as the metamyelocytes and band cells are usually included with such counts, while the reticulocytes are not, it is a larger compartment which has to be filled up in the case of the myeloid series, than in the erythroid series, consequently it may take longer time to fill it, even if there are no differences in the respective mitotic activities.

After pre- or immediate post-irradiation anoxia (venesection, altitude, etc.) an extra stimulus (erythropoietin) is added for erythroid regeneration. This, in fact, was noted by Valentine and Pearce (1952b), who attributed the rapid regeneration of the erythroid cells in venesected irradiated cats to a lower radiosensitivity of this system in respect of regeneration than the myeloid system. However, given strong enough stimulus (e.g. bacteriaemia) massive leukocytosis could be produced in fish, even after a total-body dose of 8000 r (Preston, 1959) and, after smaller doses by turpentine abscess (Cronkite and Brecher, 1955), or leukocyte antiserum (Patt *et al.*, 1955) in mammals—indicating a significant residual capacity of the marrow to respond to specific stimuli, even after relatively large doses of radiation. It is suggested that the integer fraction of the stem cells is responsible for this capacity.

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So far there is no evidence that the "available" stem cells are ever exhausted, the cause of death (and anaemia immediately prior to death) appears to be due to multiple haemorrhages initiated by gradually developing thrombopenia, and also possibly by direct capillary damage.

7. *Combating radiation damage to the marrow*

The problem of protection of haemopoietic tissue during irradiation by shielding (rat tail, hind limb, spleen), or of post-irradiation transfusion of haemopoietic tissue (bone marrow, embryonic spleen or liver) is not subject of this review, but it is clear that integer marrow cells can "re-seed", and there is evidence that clonal growth of a few (? multipotential) cells may be responsible for the re-seeding (Ford *et al.*, 1959).

However, in view of what has been said in the previous sections, the theoretical need for post-irradiation bone-marrow cell transfusion (marrow grafting) may be examined here.

In Section V it has been suggested that, after a dose of radiation a certain proportion of stem cells will receive damage. This damage is visualized as an effect on their reproductive integrity, with the result that cells enter the degenerative stage either slowly in time, or, more likely, only after one or two divisions. Although the stimulus for differentiation of stem cells into group 2 cells (pro- and basophilic normoblasts, promyelocytes) will increase with time, as the original group 2 damage results in peripheral depletion of cells, a large proportion of the mobilized stem cells (after a dose of 500 rads, 90 per cent, see Fig. 3) will be ineffective as they enter the degenerative phase after one or two divisions. Thus group 2 cell population will remain low, and, consequently the peripheral blood-cell level also. If it reaches below a certain level, and lasts a certain length of time, chance will be given for fatal haemorrhage or septicaemia. It should be remembered, however, that for the peripheral blood picture (in respect of neutrophils and platelets) to reach the maximum depression (and presumably maximum stimulation for specific stem cell differentiation) takes time. If, prior to that time, sufficient stimulation could be given to the stem cells for large numbers of them to differentiate, i.e. leave the stem cell compartment, then, because of the internal homoeostasis of the stem cell compartment, chance would be given for the *integer fraction* of the stem cell population to repopulate the compartment. Thus, by the time the platelet and neutrophil levels reach their low point, sufficient number of integer stem cells may be available for normal differentiation and blood-cell production.

According to Fig. 3, after a dose of e.g. 850 rads about 1 per cent of the stem cell population would remain integer. The usual number of

marrow cells given in post-irradiation marrow grafting is of the order of 1 per cent of the total bone marrow cellularity of the recipient animal, i.e. it would only about double the number of the recipient's integer stem cells. However, doubling the number of integer stem cells would double the peripheral output—in effect it would be equivalent to a complete single wave of division of the remaining integer stem cells. As such a complete wave may take a considerable time (days), it may be understandable that simulating it by stem cell transfusion the critical period for "bone marrow death" is shortened.

The conclusion which, nevertheless, may be drawn from the above hypothesis is that careful substitution therapy alone (e.g. platelet transfusions, prevention of septicaemia, etc.) could save an irradiated animal from "bone marrow death" until normal regeneration is completed, and that early stimulation of the stem cells for differentiation may considerably shorten the time required for such regeneration. There is evidence published in the literature which indicates that such post-irradiation treatment (e.g. venesection, hypoxia, etc.) should be considered, especially in man, as an alternative, or at least auxiliary procedure in combating radiation damage to the marrow (Schack and MacDuffee, 1949; Stohlman *et al.*, 1955; Stohlman and Brecher, 1956).

This problem is all the more relevant in man, where isologous marrow is not usually available, and autologous marrow not always practical (e.g. radiation accidents). The use of embryonic marrow has been made hazardous, quite apart from the formidable technical difficulties involved, by recent observation that even embryonic heterologous marrow may produce "secondary disease" (Crouch, 1959).

VII. Observed Effects of Tumour-chemotherapeutic Agents on the Marrow

Tumour chemotherapeutic agents can be divided into two broad groups: (1) alkylating agents, and (2) antimetabolites.

(1) Alkylating agents include such compounds as the HN2, and HN3 mustards, Nitromin, Melphalan, R.48, Leukeran (chlorambucil), Degranol, hemisulphur mustards, Myleran, triethylene melamine (TEM), triethylene phosphoramide (TEPA), and thio-TEPA. Their mode of action is not well understood; nevertheless, because of many similarities with radiation effects (mutagenic, carcinogenic, chromosome abnormalities, apart from growth inhibition), they are frequently described as "radiomimetic" agents (Dustin, 1947). The subject is excellently discussed elsewhere (*Ann N.Y. Acad. Sci.* **68,** 1958) it should be sufficient to mention only that in suitable concentrations they are powerful cytotoxic agents, cause inhibition of mitosis, chromosome abnormalities, inhibition of DNA synthesis, and extensive cell death.

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It is usually said that the lymphatic system is more affected by alkylating agents than other tissues in the body, and, consequently, they are more frequently used as tumour chemotherapeutic agents in patients with malignancy of the lymphoreticular system than other malignancies. However, this statement may be an oversimplification—it has been pointed out in the first sections of this review that the apparent sensitivity of a *tissue* may not necessarily reflect on the sensitivity of the *cells* composing it, but on the rate at which cells enter degenerative phase after injury. Secondly, some alkylating agents, e.g. Myleran, exhibit more tissue specificity for the myeloid cells than for the lymphatic cells.

(2) The antimetabolites may be subdivided into groups of folic acid antagonists (or analogues) such as aminopterin, a-methopterin; purine or pyrimidine analogues, such as 6-mercaptopurine, azaguanine, thioguanine, azapyrimidines, halogenated pyrimidines; and other miscellaneous compounds, such as urethane, azaserine, or mitomycin C. The problem of antimetabolites is also excellently reviewed elsewhere in great detail (Rhoads, 1955; Handschumacher and Welch, 1960).

Any compound which has a growth-inhibiting action would quickly show marked effects on fast-growing cell populations, or on tissues where the steady-state equilibrium depends on fast rate of cell production. Since bone marrow is an organ in which cell populations turn over very rapidly, it is understandable that cytotoxic agents show a quick and very noticeable effect on it.

Thus aminopterin (and later amethopterin) was used to produce temporary remissions in children with acute leukaemia (Farber *et al.*, 1948); it is known to be a most potent mitotic inhibitor for bone-marrow cells (Gunz, 1950), also profoundly affecting DNA synthesis in them (Lajtha *et al.*, 1955; Totter, 1955). Similarly alkylating agents have been used for the treatment of leukaemias—Myleran and chlorambucil in particular (Haddow and Timmis, 1953; Elson, 1955). The pattern of bone marrow response to these two compounds (Myleran and chlorambucil) has been very carefully investigated and compared with that obtained after radiation (Elson, 1955; Elson *et al.*, 1958), with the conclusion, that while separately both Myleran and chlorambucil show some differences from the effect produced by irradiation, the combination of the two drugs is very closely "radiomimetic". A single dose of 12·5 mg/kg of each compound given simultaneously (Myleran by mouth, chlorambucil intraperitoneally) gave a pattern of peripheral blood-cell response similar to that with a dose of 250 r whole-body X-irradiation (Elson, 1955).

It is very difficult to speculate on the similarities of actions of two agents, when the mechanism of action of neither of them is properly

understood. It is not known how irradiation affects the reproductive integrity of cells, but at least it is known that it affects a great variety of cell types in a remarkably similar fashion. No comparative dose-response studies have been performed with any of the tumour chemotherapeutic agents—it is very much hoped that use will be made of the Puck method of clonal growth of mammalian cells (Puck and Marcus, 1956) to fill this important gap in our knowledge of the action of tumour chemotherapeutic agents.

TABLE 1.

Comparison of Chlorambucil and Myleran Effect with that of X-rays on Bone Marrow Cells.

	Erythroid	*Myeloid*	*Lymphoid*
12·5 mg/kg chlorambucil	200–400 r	?(200 r)	400 r
15 mg/kg myleran	?(400 r)	400 r	200 r

Compiled from data of Elson *et al.* (1958) and Hulse (1957, 1959a,b).

The main effect of both alkylating agents and the above-mentioned antimetabolites is thought to be an interference with nucleic-acid metabolism. The alkylating agents may do this by alkylation of the phosphoryl groups of nucleic acids, the antimetabolites by blocking a specific enzyme action. However, nitrogen and sulphur mustards depress bone marrow DNA specific radioactivity only after about 8 hr, maximum depression being reached at about 24 hr (Drysdale *et al.*, 1958). As by 24 hr usually there is a profound effect on the peripheral blood, it is likely that the depression noted in DNA specific activity is a result of marrow depopulation rather than direct interference with the process of DNA synthesis. Most of the antimetabolites, however, appear to have an immediate effect on the process of DNA synthesis (Lajtha *et al.*, 1955; Totter, 1955; Prusoff *et al.*, 1956).

If a compound affects only those cells which at the time of its administration are in the process of DNA synthesis (or in any other particular stage of the cell cycle) then it is conceivable that of two cell populations the one in which the period of DNA synthesis (or the particular stage in question) occupies the longer proportion of the cell cycle will be the more affected (Fig. 9). Thus, it may be possible that with such compounds (e.g. antimetabolites) a higher proportion of the fast-growing than of the slow-growing cells are injured—unlike with radiations. If, for example, in the stem cell compartment only a small proportion of the cells are at any time in the process of DNA synthesis (while 40–60

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per cent of the group 2 cells are at any time synthesizing DNA), then a single dose of an antimetabolite should give a different type of response to a single dose of radiation: the latter, damaging an equal proportion of stem cells and group 2 cells will show the well known "initial depression–abortive recovery–second depression" pattern, while the former should only produce an initial depression followed by complete recovery. There are no such serial investigations reported in the literature, apart

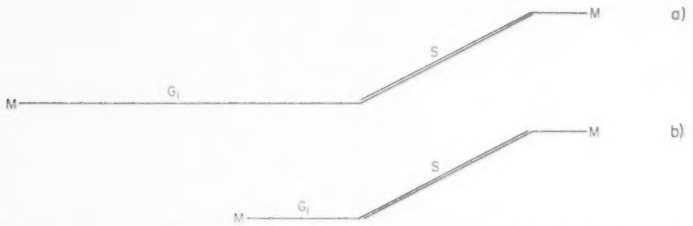

FIG. 9. Two cell cycles in which the period of DNA synthesis (or any other particular period) occupies 30 per cent (a), or 50 per cent (b) of the interphase. In a non-synchronized population therefore 30 or 50 per cent, respectively, of the cells would be at any time in the particular period.

from a study of the erythropoietic activity (^{59}Fe appearance) at different times after a single dose of 1·25 mg/kg aminopterin in rats (Lamerton and Belcher, 1957). The curve obtained is similar to that after a 200 r total-body irradiation, but the ^{59}Fe method may not be sensitive enough (unless uptake is delineated in time by postlabel "flooding" with inactive iron) to detect transient population changes.

Much more experimental observation is available on the effects of two alkylating agents—Myleran and chlorambucil. A comparative study with ^{59}Fe (Lamerton and Belcher, 1957) shows a similar response to 12·5 mg/kg chlorambucil and to 200 r total-body irradiation; the same dose of Myleran, however, gave a slightly greater effect than 400 r TBR.

Careful studies of population changes in the marrow, however, show that there are some important differences between the effects of these two alkylating agents and that of irradiation. The differences are shown in Table 1 and Figs. 10 and 11, which compare the action of the two alkylating agents with that of X-radiation. The impression clearly is that there is an element of tissue specificity, i.e. chlorambucil affects the lymphoid cells more than the myeloid cells, while Myleran affects the myeloid cells more than the lymphoid cells. The erythroid cells also appear to be more affected by Myleran than by chlorambucil. Elson and his collaborators (Elson *et al.*, 1958) in their careful analysis of Myleran and chlorambucil effects attempt to explain the differences on

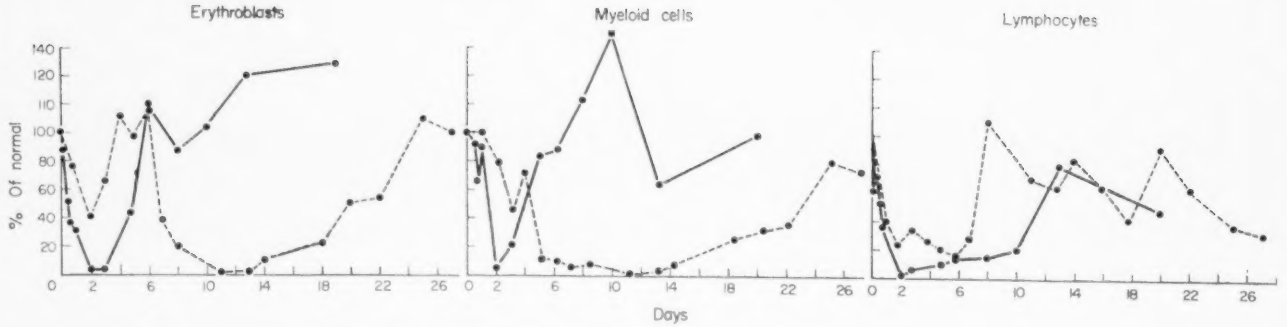

FIG. 10. Effect of a single dose of Myleran or chlorambucil on bone marrow cells in rats (after data of Elson *et al.*, 1958).

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different mechanisms of action. They suggest that chlorambucil affects cells just entering mitosis and in their DNA synthetic stage, while Myleran affects them in the G_1 period (the long pre-DNA synthetic period). Their argument is strengthened by the observation that the frequent mitotic abnormalities seen in chlorambucil-treated animals are usually absent in those receiving Myleran.

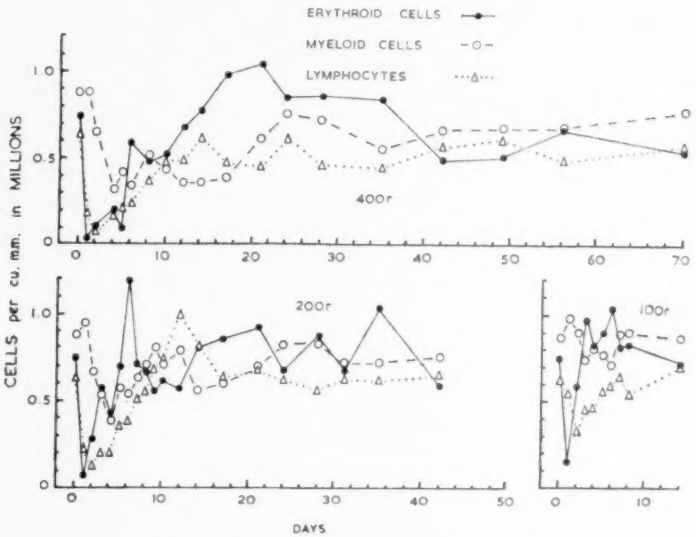

FIG. 11. Effect of a single dose of whole-body irradiation on bone-marrow cells in rats (Hulse, 1959c).

The problem, however, may be complicated also by a genuine tissue specificity, i.e. Myleran may not enter the lymphoid cells with the ease it does the myeloid and erythroid cells, and, conversely, chlorambucil may enter the lymphoid cells easier than the other cell types—thus the intracellular concentrations may differ in different cell types.

Similar situations may, theoretically, occur with antimetabolites. The efficiency of different pathways of nucleic acid synthesis may vary in different cell types, thus both the degree of uptake of an antimetabolite, and the degree of inhibition produced by it may vary independently. It is felt that, at present, all attempts to analyse the mechanism of action of tumour chemotherapeutic compounds are highly conjectural, but further investigations, with graded doses of the drugs, compared with graded dose of X-rays, may yield valuable information on their mode of action.

VIII. Problems Connected with Therapy of Leukaemias

It is well known that chronic myelocytic leukaemia responds well to treatment with radiations or chemotherapeutic drugs (e.g. Myleran). It is not at all understood, however, why it should respond to local irradiation of the spleen. Gunz has suggested that splenic irradiation may inhibit bone-marrow mitotic activity (Gunz, 1953), and recently, Lamerton has found that the rate of fall in platelets during chronic whole body irradiation (50–80 rads/day) is slower in splenectomized than in unoperated rats (Lamerton, 1960). Thus, evidence appears to be accumulating in favour of the hypothesis that irradiated spleen may be releasing an inhibitor for mitosis.

Acute (blast cell) leukaemias are not usually treated with radiations. However, there is no good theoretical reason, neither good evidence for treatment with chemotherapeutic agents being more effective than with X-rays. For example, although folic acid antagonists are frequently used, there is no good evidence that the leukaemic blast cells depend more on folic acid coenzymes than other cells in the body. The temporary remissions obtained in cases of blast-cell leukaemias may be related to the slower growth rate of leukaemic blast cells than the recovery rate of normal bone marrow elements (Lajtha, 1959; Lajtha and Oliver, 1960b).

The cardinal difference between chronic and acute leukaemias is thought to be that while in the chronic myelocytic and lymphocytic leukaemias there is, so far, no possible differentiation between "normal" and "leukaemic" cells, in most of the acute leukaemias the blast cells are highly "abnormal" in morphological appearance. However, in normal marrows the proportion of blast cells is very low (usually below 1 per cent) and in every normal marrow one may find the odd cell, which, if it occurred as 20–50 per cent of the population, would be diagnosed as a "monoblast", "paramyeloblast", "histioblast" or other "abnormal" blast cell.

It is a commonplace statement to say that the nature of leukaemia is not understood. The term "leukaemia", however, may hide a number of different pathologies. While a better understanding of the mechanism of radiation effects on the bone-marrow cells may improve our ability to combat radiation damage, it is the chemotherapy of malignancies to which hopes of a rational therapy of leukaemia must be attached.

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SOME FACTORS INFLUENCING THE DISPERSION OF INDICATOR SUBSTANCES IN THE MAMMALIAN CIRCULATION

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SOME FACTORS INFLUENCING THE DISPERSION OF INDICATOR SUBSTANCES IN THE MAMMALIAN CIRCULATION

P. I. Korner

I. Introduction

Just over 60 years ago G. N. Stewart first suggested that the output of the heart could be determined from analysis of the dilution of an indicator substance injected into the circulation. Some 30 years elapsed before the careful studies of Hamilton and his colleagues firmly established the validity of the indicator dilution principle for the measurement of cardiac output. They provided a clear description of the shape of the arterial time–concentration curve obtained after the rapid injection of an indicator substance on the venous side of the circulation (Kinsman, *et al.*, 1929; Moore, *et al.*, 1929; Hamilton *et al.*, 1932). Their work extended the application of the indicator dilution principle since they showed that the volume between injection and sampling sites* could be calculated from the product of the cardiac output and the mean circulation time of the arterial time–concentration curve.

The development of methods for the continuous recording of the dilution curves has resulted in closer study of the dispersion of indicator. The discovery of abnormal dispersion patterns in a variety of circulatory disorders (e.g. central vascular shunts, incompetence of the heart valves) has provided the chief stimulus for the study of dispersion. Interest in the abnormal dispersion patterns has overshadowed, not unnaturally, a study of the significance of normal dispersion patterns in relation to the underlying hydraulic processes of circulatory mixing. Since the pathways traversed by the blood are complex and the type of flow varies in different parts of the circulation, theoretical treatment of these processes seems at present not possible and the study of the dispersion of indicator substances in the circulation has not advanced beyond the descriptive stage.

The methods used in the calculation of flow and volume form a convenient introduction to the study of the dispersion of indicator

* Volume between injection and sampling sites is always referred to as volume.

substances. These are only briefly considered in the next section of this article and a much more comprehensive discussion is available in the excellent review by Dow (1956). The remaining sections give an account of some normal and abnormal dispersion patterns and an attempt is made to relate the observed effects in a qualitative fashion to the underlying hydraulic processes.

II. Measurement of Flow and Volume

1. *Methods*

The indicator material can be injected either by rapid injection or by continuous infusion. The former method is more commonly employed. Hamilton and Remington (1947) pointed out that the time–concentration curve obtained after rapid injection was the first derivative of the curve obtained by the use of the continuous injection method. Both methods give identical estimates of flow and volume though the exact recirculation point is more difficult to establish with the latter method (Howard *et al.*, 1953; Peterson *et al.*, 1954; Shepherd *et al.*, 1955). The indicator materials in common use include the protein-bound dyes T–1824, indigo carmine and indocyanine; radioactively labelled plasma proteins; red cells labelled with ^{32}P or ^{51}Cr (Dow, 1956; Lacy *et al.*, 1955; Fox *et al.*, 1956; McIntyre *et al.*, 1951). The dye indocyanine and the radioactive indicators have the advantage that they can be used in the presence of a fluctuating oxygen saturation. It is important that there should be no loss of material between the injection and sampling site and this excludes diffusible substances such as strong salt solution or ^{42}K except in cases when the injection site is on the left side of the heart (e.g. Conn, 1954).

Continuous recording of the time–concentration curves has effectively superseded the older discontinuous sampling methods. A summary of the various densitometers for use with coloured dyes, and of the radioactive counters is given by Dow. In most devices the sampling site is connected to the recording instrument by means of a catheter and blood is drawn through this at a constant rate. It is important that the distortion due to this catheter-sampling system should be kept at a minimum, and this problem has recently become even more acute as a result of the use of venous sampling sites which require a long catheter system.

The effects of the sampling system on the degree of curve distortion have been studied in a quantitative manner only relatively recently (Fox *et al.*, 1957; Lacy *et al.*, 1957; Sheppard, 1957; Sherman *et al.*, 1959; Edwards *et al.*, 1959; Cheesman *et al.*, 1959; Gonzalez-Fernandez *et al.*, 1959). The degree of curve distortion is minimized by suitable arrangement of the mechanical components of the system. An increase

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in flow rate through the catheter system, a decrease in the length and the diameter all help to improve the dynamic response of the detecting instrument. Some improvements in the design of the cuvette and of electrical components of the densitometer have also recently taken place, the latest instrument developed at the Mayo Clinic having a 90 per cent deflexion time of less than 0·05 sec, in contrast to that of most other instruments of 0·2–1·0 sec. The indicator dilution curves are relatively slow rising and falling curves and very fast responses are usually not required. Sherman *et al.* have suggested a figure of merit for the adequacy of a given catheter-sampling system; the volume of this system divided by twice the flow rate should be less than the time in which significant changes in indicator concentration can occur, i.e. the interval between successive heart beats. In the study of the dispersion of indicator it is obviously essential to minimize the effect of curve distortion; whilst it cannot be completely eliminated, the use of a standard catheter-instrument system for a variety of experimental procedures will at least ensure that the degree of curve distortion is relatively constant.

The question of curve distortion has been attacked in a different way by Gonzalez-Fernandez *et al.* These workers make due allowance for the distortion introduced by the system, and have devised a numerically recursive method to recover the true "input" curve at the arterial sampling point. The dynamic response of a given catheter-sampling system is determined experimentally. Knowing the characteristics of this system the "input" curve is then recovered from the distorted curve as the solution of an integral equation of the convolution type. They have shown that curves recorded simultaneously from catheter-sampling systems of widely differing responses yielded the same "input" curve when treated in this way (Cheesman *et al.*, 1959). The method requires the use of a digital computer, and though it is the best attack on this problem to date, care in the arrangement of the hydraulic components of the system is probably sufficient for most practical purposes.

2. *Description of the indicator dilution curve*

Figure 1 (a) shows a time–concentration curve recorded from the femoral artery of a normal dog, following the rapid injection of the blue dye T–1824 into the lower part of the superior vena cava. There is a delay following the injection before the first particles of indicator appear at the sampling site (appearance time). The dye concentration then rises rapidly to a peak concentration value and subsequently returns more slowly towards the baseline. The downslope of the curve is interrupted at the point R and the subsequent secondary rise in

dye concentration is due to recirculation of indicator particles that have completed their first passage through the circulation.

In calculating flow and volume it is necessary to know the full time course of the first passage of indicator through the circulation. Since this is obscured by recirculation, extrapolation of the downslope of the primary curve is required. Hamilton and his colleagues showed in circulation models, where recirculation was prevented, that the downslope was exponential. Plotting the logarithm of the concentration

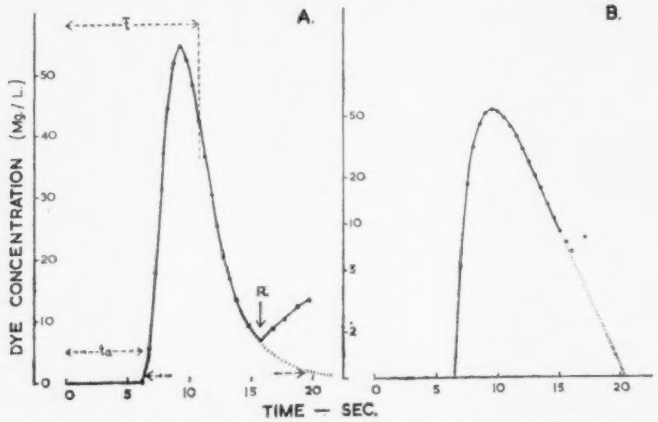

FIG. 1. (a) Indicator dilution curve from a normal dog; t_a is the appearance time and $\bar{t}$ is the mean circulation time. Indicator is injected at zero time. R denotes commencement of recirculation and the arrows at the blood concentration value of 1 mg/l show the limit of extrapolation for the calculation of the variance.

(b) Same curve illustrating Hamilton's method for extrapolating the downslope.

values of the downslope against time, once the downslope has been clearly established, results in a straight line. This extrapolation is also useful in indicator dilution curves obtained from animals (Fig. 1b) and generally permits adequate reconstruction of the primary curve.

3. *Measurement of flow, using the Hamilton extrapolation*

If a quantity of indicator is rapidly injected into the venous part of the circulation at time $t = 0$ and the time–concentration curve obtained from a peripheral artery then the flow (cardiac output) is given by

$$F = 60\, I / \int_0^\infty c(t)\, dt \qquad . \quad . \quad . \qquad (1)$$

where F = flow rate (l/min);
I = quantity of indicator injected;
$c(t)$ = concentration of indicator at time t sec.

In other words the flow rate is calculated from the amount of indicator injected and the area of the extrapolated curve.

The following assumptions are implicit in the calculation of flow (and also of volume) from equation (1):

(1) The distribution of traversal times for fluid particles entering the system does not change with time ("stationarity" of flow).

(2) The distribution of traversal times for the indicator must be representative for the whole fluid.

(3) There is no loss of indicator material, or sequestration, between injection and sampling sites.

(4) There must be sufficient turbulence in some section of the system between injection and sampling sites, so that the concentration of indicator leaving this part of the system is reasonably uniform (Rossi *et al.*, 1953).

(5) The semilogarithmic extrapolation accounts adequately for the primary curve.

Condition (1) merely states that the system must be in a steady state and is common to all indirect cardiac output methods.

Condition (2), requiring that the distribution of traversal times of indicator must be representative for the whole fluid appears at first sight to invalidate the method in the case of blood. Meier and Zierler (1954) have pointed out in this connexion, that the large vessel haematocrit ratio measures the ratio of red cell *flow* to total blood flow, and not the ratio of red cell volume to total blood volume. The total blood flow can thus be calculated from the plasma or red cell flow by means of a simple haematocrit conversion factor. Given the plasma flow F_P and the large vessel haematocrit Ht, the total blood flow equals $F_P/(1—Ht)$. The determination of cardiac output in the same animal using red-cell labels as well as plasma labels gives identical estimates of total blood flow (Lawson *et al.*, 1952; Rapaport *et al.*, 1956; Groom and Rowlands, 1958).

The use of red-cell labels and protein-bound indicators minimizes loss from the pulmonary circulation except through sequestration effects. It is unlikely that the latter play a significant role in the normal mammalian circulation. In circulation models Thorburn *et al.* found that an overestimate of the true flow due to sequestration of indicator occurred when injection was carried out just upstream from a large rigid chamber, but that this effect was no longer observed when an elastic chamber of equal dimensions was used instead; evidently the pulsation of the elastic walls with each cycle prevented the excessively slow clearance of indicator from parts of the chamber.

When the indicator is injected upstream from the left ventricle,

sufficient turbulence occurs in this chamber to conform with condition (4). Beard *et al.* (1951) have obtained the same estimates of cardiac output in curves recorded simultaneously from a number of peripheral arteries, indicating reasonable uniformity of distribution of indicator material in the various arterial branches. Attempts have been made, however, to apply the indicator dilution principle to the study of regional blood flow. Andres *et al.* (1954) tested this possibility for the measurement of forearm blood flow. Indicator was injected into the brachial artery and sampling was carried out from two antecubital veins. They endeavoured to produce a uniform distribution of indicator by injecting dye through a jet orifice of 25 μ diameter, thereby creating turbulence at the injection site. The method did not even then give reliable results and there was an irregular distribution of indicator in the veins draining the forearm. Similar findings were observed by Nylin and Blömer (1955) and Nylin and Hedlund (1958) when applying the method to the measurement of cerebral blood flow. The reason for the non-uniformity of distribution of indicator will become apparent in Section IV. The results indicate that the indicator-dilution method is not well suited to the measurement of peripheral blood flow.

The adequacy of the Hamilton extrapolation method has been extensively tested in circulation models (Kinsman *et al.*, 1929; Thorburn *et al.*, 1959). No significant difference between calculated flow and independently measured flow was observed when the concentration values were extrapolated to infinity; the standard deviation of a single measurement was about 3–4 per cent. In the normal mammalian circulation the indicator-dilution method has been compared with the direct Fick method and different types of flowmeter, and over a large number of series no systematic or significant difference has been found in the results obtained by the two methods (Dow, 1956). Some individual series have, however, shown slight variations in either direction. No differences in estimates of cardiac output are observed when the curves are obtained after injection in different injection sites (Hetzel *et al.*, 1954). The evidence in general supports the view that the semi-logarithmic extrapolation gives an adequate estimate of the area of the curve.

4. *Measurement of volume*

The indicator-dilution curve may be considered as a frequency-distribution curve, where the concentration values represent the frequency of indicator particles passing the sampling point of time t. The average time of passage of indicator particles is termed the mean circulation time, and is calculated from the first moment of the frequency function.

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$$\bar{t} = t_a + \frac{\int_{t_a}^{\infty} t\, c(t)\, \mathrm{d}t}{\int_{t_a}^{\infty} c\,(t)\, \mathrm{d}t} \quad . \quad . \quad . \quad (2)$$

where $\bar{t}$ = mean circulation time (sec);
t_a = appearance time (sec);
$c(t)$ = concentration of indicator at time t.

The volume between injection and sampling sites is given by the product of the flow and the mean circulation time:

$$V = F\bar{t}/60 \quad . \quad . \quad . \quad (3)$$

where V = volume (l.);
F = flow (l./min).

For a detailed derivation of equation (3) the paper of Meier and Zierler (1954) should be consulted. All the conditions necessary for the calculation of flow also apply in the calculation of volume, but some of these require further comment.

The volume calculated from equation (3) has the greatest anatomical meaning if the system between injection and sampling sites is a "closed" one, i.e. with a single inlet and outlet. In the mammalian circulation this requires an appropriate choice of injection and sampling sites. Suitable injection sites include points lying between the great veins near the right atrium and the left ventricle. The only sampling site that fulfils the above requirements is near the root of the aorta. If the sampling site is at a more peripheral artery, say the brachial or femoral artery, the calculated volume includes all parts of the arterial system having a similar distribution of traversal times as the artery used for sampling (Meier and Zierler, 1954). Since the arterial volume is a relatively smaller fraction of the total blood volume than the venous volume, choice of a peripheral artery does not result in a large discrepancy in the calculation of the "central" blood volume; on the other hand, choice of a peripheral vein as the injection site will result in the inclusion of a substantial proportion of the venous blood volume in addition to the central volume (Hetzel *et al.*, 1954).

The accuracy of the estimation of volume from equation (3) has been checked in water-filled model systems. The results of calculated volume agreed with direct measurement in models differing widely in their design and the standard deviation of a single estimate varied from 3–5 per cent (Kinsman *et al.*, 1929; Hoffman and Shillingford, 1957; Thorburn *et al.*, 1959). In animal experiments the estimates of volume obtained from equation (3) have been checked by Schlant *et al.* (1959).

The central blood volume from the right atrium to the ascending aorta was calculated from an indicator-dilution curve using the plasma label T–1824. The dogs had previously had their red cells labelled with ^{51}Cr and the actual amount of blood in the heart and lungs was calculated from the total radioactivity in the blended homogenates of these organs. There was a good correlation between the two estimates, though the volumes calculated from the dye curves were about 10 per cent above the actual blood volumes throughout the range of observations. The most likely reason for the difference is the lower haematocrit ratio in the smaller pulmonary blood vessels (Dow *et al.*, 1946; Rapaport *et al.*, 1956; Lawson *et al.*, 1952). Differences in the haematocrit ratio of various tissues and the large-vessel haematocrit ratio are well recognized. The ratio of total red cell volume to total red cell volume plus total plasma volume are significantly below the large vessel haematocrit when red-cell volume and plasma volume are determined independently (Gibson *et al.*, 1946; Chaplin *et al.*, 1953; Gregersen and Rawson, 1959). This relationship varies in different tissues and depends on axial-stream formation of red cells in the minute vessels.

In the calculation of total blood flow from the plasma or red cell flow a simple haematocrit conversion factor could be validly applied. In the estimation of volume this is not suitable since the mean circulation time for plasma is greater than that for red cells (Dow *et al.*, 1946; Freis *et al.*, 1949; Lawson *et al.*, 1952; Groom and Rowlands, 1957). When using only the plasma mean-circulation time the blood volume is overestimated, as indicated by the results of Schlant *et al.*

In order to calculate the true "central" blood volume, the use of both red cell and plasma labels is required. Under these conditions the blood volume between injection and sampling sites is given by

$$V_B = V_P \left[1 + \frac{\bar{t}_R \, Ht}{\bar{t}_P \, (1 - Ht)} \right] \quad . \quad . \quad . \quad (4)$$

where V_B = central blood volume;
V_P = central plasma volume;
$\bar{t}_R$ = mean circulation time for red cells;
$\bar{t}_P$ = mean circulation time for plasma;
Ht = large vessel haematocrit ratio.

Chaplin *et al.*, have shown in man that the body haematocrit is about 92 per cent of the large vessel haematocrit over a wide range of values. In view of the fairly constant relationship between the two values they suggest that the body haematocrit can be estimated from the large-vessel haematocrit with reasonable precision. The results of Schlant

et al., and of Rapaport *et al.* in the dog, suggest that the pulmonary haematocrit bears an approximately similar relationship to the total-body haematocrit. The use of a constant correction factor is probably reasonable in man or the dog, but in cats Groom and Rowlands (1958) found that there was great variation between animals in the value of the ratio of body haematocrit/large-vessel haematocrit. Under these conditions an arbitrary correction seems unsuitable and the central blood volume must be determined by means of two indicator substances.

III. Dispersion of Indicator in the Normal Circulation

1. *Estimation of dispersion*

In the calculation of flow use is made of the area of the curve, or total number of indicator particles, while the first moment of the frequency function is utilized in the calculation of volume. The study of the dispersion of indicator in the circulation requires additional parameters to specify the distribution in more detail. The asymmetrical nature of the frequency function has undoubtedly retarded application of the logical parameters for the estimation of dispersion. Most workers characterize the dispersion of indicator by a variety of time ratios of different parts of the curve or by the downslope. (Nylin and Celander, 1950; Newman *et al.*, 1951; Broadbent and Wood, 1954; Dow, 1954; Hetzel *et al.*, 1954; Korner and Shillingford, 1955; Woodward *et al.*, 1957a). These measurements are all fairly highly correlated with one another (Dow, 1954). However, from the point of view of specifying the frequency function these measurements, though useful, cannot be regarded as optimal parameters.

Korner and Shillingford (1956) found that the second moment of the frequency function, or the variance of the curve, was a satisfactory measure of the dispersion of indicator. In order to avoid excessive bias in the calculation of the variance due to the long tail of the curve (Fig. 1a), they extrapolated the curve to an arbitrary concentration value of about one-fortieth of the peak concentration (shown by the arrows in Fig. 1a). This results in a systematic error of about 4 per cent in the calculation of flow and volume (Thorburn *et al.*, 1959). The use of the variance as a statistic of dispersion can be regarded as a first step in the specification of the frequency function; it will become apparent that it does not specify the function fully and that the asymmetry of the curve must also be taken into account. Since the distribution is asymmetrical one would not expect that the variance would have the properties of the variance of a Gaussian distribution. However, in the application of this statistic to a number of practical problems,

deviations from the properties of a Gaussian variance are often surprisingly small.

The variance of the curve is calculated from the following relationship

$$V_x = 1/S(c)\,[S\,(ct^2) - S^2(ct)/S\,(c)] \quad . \quad . \quad . \quad (5)$$

where V_x = variance of the curve (sec^2);
c = indicator concentration;
t = time (sec) after commencement of curve;
S = summation of the bracketed term.

2. *Dispersion of indicator in straight tubes*

The dispersion of indicator substances has been studied in straight tubes during steady laminar or turbulent flow of water.

When indicator material is introduced rapidly into a tube where the flow is laminar, the indicator material becomes distributed in relation to the paraboloid velocity profile. A dilution curve obtained some distance from the injection site shows a very marked degree of asymmetry and a long tail (Rossi *et al.*, 1953; Sheppard, 1954).

When the flow in the tube is turbulent, the indicator curve is symmetrical and Gaussian (Rossi *et al.*, 1953; Taylor, 1954). Taylor has shown that the distribution is centred about the point $\bar{u}\,t$, where $\bar{u}$ is the mean velocity flow and t the time after injection of indicator. When the sampling site is less than about 100 tube diameters from the injection point the distribution is not Gaussian, but is asymmetrical; Taylor has suggested that the laminar boundary layer contains an appreciable quantity of indicator which diffuses relatively slowly into the central layer. When the distance between injection and sampling point exceeds 100 tube diameters, mixing of the two components becomes complete and the distribution becomes Gaussian; with relatively low Reynolds numbers even greater distances are required to observe this effect. Taylor has thus shown both theoretically and experimentally that when there is relatively little radial variation in indicator concentration the distribution ultimately becomes Gaussian. His work has definite application to the distribution of indicator substances in the mammalian circulation.

3. *Dispersion of indicator in more complex models*

3.1. *Description of the model.* These models consist of a more complicated arrangement of tubes and the dispersion of indicator has been studied during pulsatile flow. The advantage of these systems lies in the fact that a greater degree of control over the experimental variables can be achieved than is possible in the mammalian circulation, and they are useful in the initial definition of the problem (Korner and Shillingford, 1955; Thorburn *et al.*, 1959).

Figure 2 is a diagram of the model used by Thorburn *et al.* The "venous" system consisted of rigid glass tubing of about 2 cm diameter; the rigid section R could be replaced by a thin elastic segment E of somewhat larger volume. Pulsatile flow was provided by a piston pump; an annular entrance and exit orifice into the pump chamber was used in order to allow good mixing of indicator in the "ventricle", and unidirectional flow was maintained by means of metal valves. The

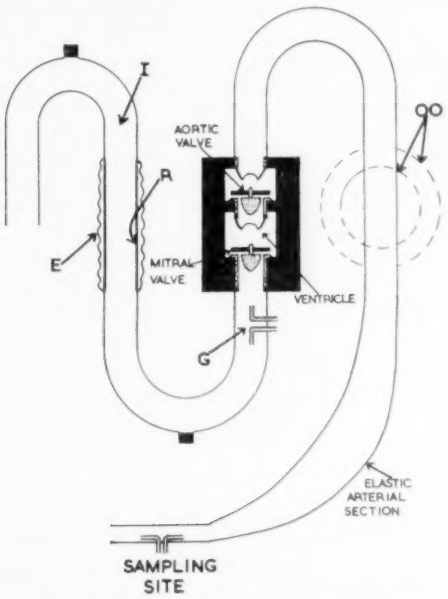

FIG. 2. Schematic diagram of circulation model. The rigid section R could be replaced by wider-bore elastic tubing E, and the volume of the arterial system could be increased by substituting the concentric double sphere OO for a section of the arterial system. I and G represent different injection sites. (From Thorburn *et al.*, 1959.)

"arterial" system consisted of about 40 cm of rigid tubing and the remainder was wide-bore elastic tubing. The volume of this portion of the system could be increased by incorporating a chamber consisting of two concentric glass spheres (OO). The passage of dye through this chamber was orderly and progressive, in contrast to the swirling observed during its passage through a single large chamber.

3.2 *Effect of flow and volume on the dispersion of indicator in a system of constant path length.* Indicator was injected at point I (Fig. 2) and the time–concentration curve recorded from a distant sampling site. Flow and volume could be varied independently of one another in this

system. Streaks of dye were observed during passage of indicator through the "venous" system, though there was a tendency for greater mixing as the fluid passed the various tube junctions. In the "ventricle" flow was turbulent.

The effect of changes in the flow and volume on the dispersion of indicator are shown in Figs. 3 and 4. When the volume was maintained

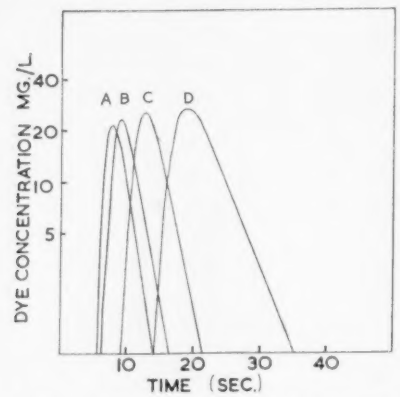

FIG. 3. Replotted indicator dilution curves obtained in the circulation model. The volume was maintained approximately constant at 600 ml. Flow $A = 3{\cdot}9$ l./min; $B = 3{\cdot}3$ l./min; $C = 2{\cdot}1$ l./min; $D = 1{\cdot}3$ l./min.

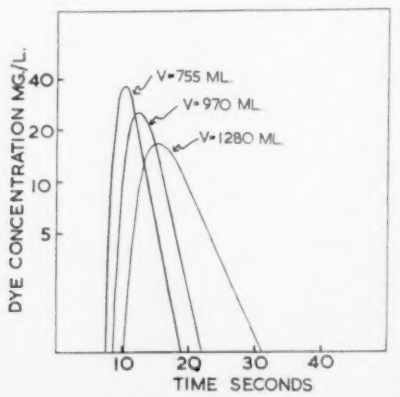

FIG. 4. Replotted indicator dilution curves obtained in the circulation model. The flow was maintained constant at 3·9 l./min and the volume was varied as shown.

constant, increase in the flow resulted in diminished dispersion of the indicator (Fig. 3); the appearance time was shorter, the dye concentration rose more sharply to a peak value and the downslope was steeper. In the examples shown in Fig. 4 the flow was maintained constant and

the volume altered. The dispersion of indicator increased as the volume became larger; the appearance time was slightly prolonged, the dye concentration reached its peak value more slowly and the downslope was less steep.

TABLE 1

Regression Equation Relating the Variance of the Dye Curve (sec²) *to Flow* (ml/min) *and Volume* (ml) *in a System of Constant Pathlength in the Circulation Model (Fig. 2); Analysis of Variance Shows the Small Magnitude of the Residual Error Term after Calculating the Regression Function*

Regression equation	log V_x = 0·3130 −2·108 log flow +2·736 log volume			
Source of variation	*D/F*	*Sum of squares*	*Mean square*	*F*
Regression function	2	4·880	2·440	1015*
Error	33	0·079	0·0024	
Total	35	4·959		

Standard error of a_1 = 0·049.
Standard error of a_2 = 0·101.
D/F = degrees of freedom.
* Highly significant relationship.

Since flow and volume can be varied independently, the relationship of these factors to the variance of the curve (Vx), can be expressed by calculating a multiple-regression equation from the data. The most suitable equation has been found to be of the type:

$$\log V_x = K - a_1 \log \text{flow} + a_2 \log \text{volume} \quad . \quad . \quad .(6)$$

where K = intercept value of the regression function;
a_1 = partial regression coefficient due to flow;
a_2 = partial regression coefficient due to volume.

In the above type of system this regression function accounts for practically all the initial variation in the variance of the curve (Table 1). The relationship of the variance to the flow is shown at three separate levels of volume in Fig. 5(a). The slope of the line represents the partial regression coefficient for flow, and the location of each line is determined by the mean volume level for each set of points. The slight divergence of the observed points from the calculated lines at high and low flows is due to the changes in volume in the elastic portions of the system and is taken into account in the regression function of Table 1.

Figure 5(b) illustrates the relationship of the variance to the volume at three separate levels of flow.

The fact that the regression function accounts for practically all the variation in the dispersion of indicator implies that the asymmetry of the curves must remain approximately constant under the experimental conditions, which include a threefold variation in the flow and a twofold variation in volume.

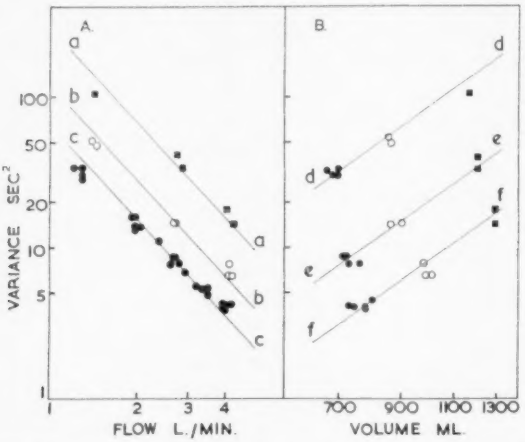

FIG. 5. (a) Relationship of variance (sec²), to flow (l/min) in normal indicator–dilution curves obtained in the model at three different mean levels of volume. The lines *aa*, *bb*, *cc*, are the calculated regression lines, with each mean volume level maintained constant. The slope of the lines is the partial regression coefficient for flow (Table 1).

(b) Relationship of variance (sec²), to volume (ml) at three different mean levels of flow. The lines *dd*, *ee*, *ff*, are the calculated regression lines with each mean flow level maintained constant. The slope of these lines represents the partial regression coefficient for volume (Table 1). (From Thorburn *et al.*, 1959.)

It can be seen from Table 1 that the partial regression coefficients differ significantly from each other. At constant volume the variance of the curve varies approximately as the square of the flow, i.e. the standard deviation of the curve varies inversely as the mean velocity of flow through the system. Since the path length was constant, volume could only be altered by changing the mean cross-sectional area of the system. Thus the increase in dispersion observed when increasing the volume (at constant flow) is partly the result of a lowering of the mean velocity of flow, and partly due to the increase in cross-sectional area available for dilution. The difference in the values of the partial

regression coefficients is thus due to the fact that they represent somewhat different processes in the dispersion of indicator.

3.3. *Variations in the dimensions of the system.* By varying the injection site the dispersion of indicator can be compared in a number of systems of varying path length as shown in Fig. 6. The volume within any system of constant path length could be varied, so as to fall in the range of the other systems. When there is some overlap in total volumes the dispersion can be compared in different systems at constant flow

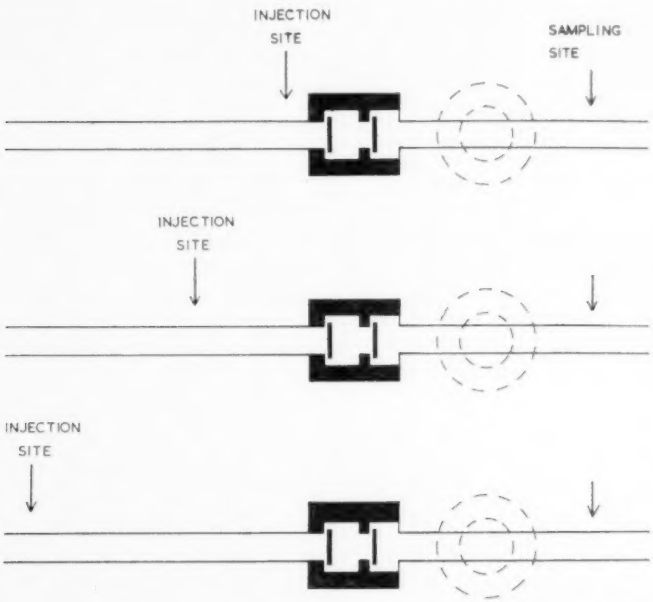

FIG. 6. Illustrating the method of changing the distance between injection and sampling sites used in the circulation model. Each arrangement of constant path length is termed a "system", and the volume between injection and sampling sites is varied within each system to fall in the range of the other systems.

and volume. The dimensions of two systems compared under these conditions are clearly different in regard to the distance between injection and sampling sites and the mean cross-sectional area. The systematic differences in the dispersion are shown in Fig. 7. The flow and volume was constant, and curve *R–E–OO* was obtained from a relatively short path length system while curve *S* was obtained from a system whose path length was 25 cm longer. The dilution curve obtained from the latter system had a longer appearance time, a

somewhat higher peak concentration and a sharper downslope. The mean circulation time was the same and the curve was more symmetrical than curve *R–E–OO*. Thorburn *et al.* have shown that in

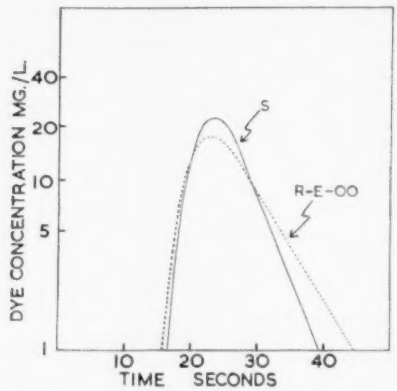

FIG. 7. Replotted dye-dilution curves obtained from two systems of different path lengths. Curve *R-E-OO* was obtained from a system in which the distance between injection and sampling sites was 150 cm; the system from which curve *S* was obtained had a path length of 175 cm. The flow was 2·65 l/min and the volume 1150 ml in both experiments. (From Thorburn *et al.*, 1959.)

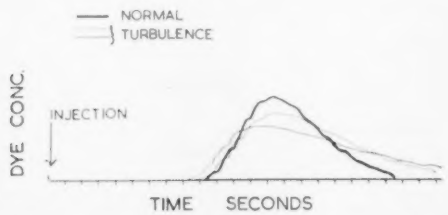

FIG. 8. The effect of turbulence in a large elastic chamber on the contour of the indicator dilution curve. The curves have been retraced from the original records. The heavy-lined curve was obtained when all parts of the model except the "ventricle" were tubes as shown in Fig. 2: the dotted curves were obtained when section *R*, or part of the arterial tubing was replaced with a large elastic chamber. The flow and volume were approximately the same in all curves. (From Thorburn *et al.*, 1959.)

systems of different path lengths, the multiple regression equations relating the variance of the curve to flow and volume in each system, all have very similar partial regression coefficients, but differ significantly in their intercept values.

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Studies of the contribution of individual components of the volume in a given system to the total variance of the curve have shown that the indicator is less dispersed in a long, narrow segment of tubing than in a short, wide segment of equivalent volume (Thorburn *et al.*). This is a qualitatively similar effect to that observed in systems of different dimensions, and suggests that different sections of volume between injection and sampling site may contribute unequally to the variance of the curve. When large elastic chambers were incorporated in the system the flow in these chambers became turbulent. The dispersion of indicator increased, when compared to the dispersion observed in a system of equivalent path length, flow rate and volume where the type of flow was more regular (Fig. 8); the partial regression coefficients of the multiple-regression equations were again similar to the standard systems and differed significantly only in their intercept values.

The systematic differences in the intercept values of the various regression equations seemed to be related to dimensional differences or differences in the flow pattern of the various systems and also to changes in the degree of asymmetry of the curves. The asymmetry was greatest in the short path length systems and diminished as the distance between injection and sampling site increased (compare the normal curves in Fig. 17). The systematic differences in the dispersion of indicator observed under the various experimental conditions suggest that the variance of the curve is only effective in specifying the curve *within* a given system, but does not fully describe the systematic changes in dispersion *between* the various systems. This suggests that additional parameters must be determined to account for these differences.

4. *Dispersion of indicator in the mammalian circulation*

4.1. *Distance between injection and sampling sites.* Some of the factors influencing the dispersion of indicator in the dog were studied by Edwards and Korner (1958) and by Korner *et al.* (1960). They attempted to specify the distribution of indicator in the normal circulation with sufficient accuracy to form a basis for the quantitative estimation of valvular incompetence. The sampling site in all experiments was the femoral artery, and injection of dye was made into the pulmonary artery, the right ventricle and the venae cavae. The animals were studied on a number of different occasions, so that multiple-regression equations could be derived for individual animals. Variation in flow rate and volume was produced in each animal by means of bleeding.

Comparison of the multiple-regression equations derived from animals with a normal blood volume, with those obtained after bleeding

showed only very slight difference in the values of the partial regression coefficients and intercept values. This suggests that reduction in the blood volume had little effect on the distribution of pathways traversed by the indicator through the central circulation.

When the injection site was moved from the pulmonary artery to the vena cava just upstream from the atrium there was a significant alteration in the intercept values of the equations, with no significant change in the partial-regression coefficients (Table 2). *For a given flow and volume*, the indicator was less dispersed when the distance

TABLE 2

*Regression Equations Relating the Variance of the Curve to Flow and Volume**

(The regression equations in part A of the table were calculated from the pooled data obtained from twenty normal dogs, for each injection site. Analysis of variance† showed that there was no significant difference in the partial regression coefficients in any of the equations, but that there were significant differences in intercept values. Part B of the table shows the best fitting regression equations based on the complete analysis of variance.)

	Injection site	*No. of curves*	*Regression equation*
A	P.A.	31	$\log V_x = 3{\cdot}7296 - 2{\cdot}2622 \log \text{flow} + 1{\cdot}8235 \log \text{volume}$
	R.V.	46	$\log V_x = 3{\cdot}4778 - 2{\cdot}1647 \log \text{flow} + 1{\cdot}7748 \log \text{volume}$
	S.V.C.	29	$\log V_x = 3{\cdot}5126 - 2{\cdot}4792 \log \text{flow} + 2{\cdot}1380 \log \text{volume}$
B	P.A.	31	$\log V_x = 3{\cdot}6139 - 2{\cdot}2899 \log \text{flow} + 1{\cdot}9041 \log \text{volume}$
	R.V.	46	$\log V_x = 3{\cdot}5612 - 2{\cdot}2899 \log \text{flow} + 1{\cdot}9041 \log \text{volume}$
	S.V.C.	29	$\log V_x = 3{\cdot}4894 - 2{\cdot}2899 \log \text{flow} + 1{\cdot}9041 \log \text{volume}$

P.A. = pulmonary artery.
R.V. = right ventricle.
S.V.C. = superior vena cava.

* Data from Korner, *et al.* (1960)
† Described by Edwards and Korner.

between injection and sampling site was greatest. This is in agreement with the observations made in the circulation model. It may seem that this presentation of the results is somewhat artificial; however, their meaning can best be appreciated from a numerical example. In a particular dog the flow rate was 3000 ml/min and the calculated volume between pulmonary artery and femoral artery was 500 ml.

The variance of the curve, from the pulmonary artery equation of Table 2B, was 6·17 sec^2. After moving the injection site to the superior vena cava the flow rate remained at 3000 ml/min and the calculated volume was 590 ml. The variance of the curve from the superior vena cava equation was 6·35 sec^2. If the volume had increased by the same amount when injecting into the pulmonary artery as a result of expansion of the pulmonary vascular bed, the expected variance from the pulmonary artery equation would be 8·46 sec^2. The observed change in variance is much smaller than would be expected for an equivalent increase in the volume of the short pathlength system.

When the distance between injection site and sampling site increases through the central circulation the curves become more symmetrical. The results of Edwards and Korner and others were all obtained after injection on the right side of the central circulation, and Dow has shown that the asymmetry of the curves is greater in curves obtained after left ventricular injection than after pulmonary artery injection. The observations of Hetzel *et al.* (1954) indicate, however, that the asymmetry of the curves increases again when indicator is injected into a peripheral vein; this apparent anomaly is discussed in detail in Section IV.

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The analysis of the downslope of the curve in different injection sites, by Pearce *et al.* (1953) showed very slight differences in this measurement after pulmonary-artery injection and right-atrial injection. This finding is similar to the results in the above example and has resulted in the suggestion that the pulmonary vascular bed made the greatest contribution to the dispersion of indicator in its passage from the venous system to the arteries. This interpretation of the experimental findings tends to emphasize the importance of dispersion in specific anatomical regions. It seems likely that this view is qualitatively correct; however, the quantitative contribution of the pathways of specific anatomical regions is more difficult to assess than might appear at first sight when the distance between injection site and sampling site is altered. The exact contribution can only be assessed if the degree of asymmetry remains constant. The degree of asymmetry diminishes as the distance between injection and sampling site increases and it will be seen in Section IV that this is the result of fundamental rearrangement of the indicator distribution. It seems clear that this factor is also partly responsible for the relatively slight increase in the variance of the curve when the injection site is moved upstream in the central circulation, and it seems better not to over emphasize at this stage, the quantitative contributions of specific anatomical pathways. This is in agreement with the interpretation of the results in the model; moving the injection site upstream resulted in a more symmetrical

distribution, without particular alteration in the basic structure of the individual components of the system. The systematic differences in dispersion were of the same order of magnitude in the dogs as in the circulation model for an equivalent change in distance between the injection sites.

4.2. *Differences between individuals.* The regression equations in Table 2 were derived from the pooled data for each injection site obtained from twenty dogs. Edwards and Korner observed significant differences in the intercept values of the regression equations *between* different animals but did not fully appreciate the quantitative importance of this finding. The more extensive subsequent studies by Korner *et al.* (1960) showed that there were great differences in dispersion between animals for a given flow and volume, using a particular anatomical injection and sampling site. This is illustrated in an example from their data in Table 3. Assuming the flow to be 3000 ml/min and

TABLE 3

Regression Equation Relating Variance of Indicator Dilution Curve to Flow and Volume

(The equations were obtained from pulmonary artery injection. There was no significant difference in partial regression coefficients, but only in the intercept values, and the equations are accordingly the best fitting regression equations based on the complete analysis of variance. The difference in intercept values represents the maximum difference encountered when using curves for a given injection and sampling site.)

Dog	*Best-fitting regression equation*
A	$\log V_x = 3{\cdot}7669 - 2{\cdot}2899 \log \text{flow} + 1{\cdot}9041 \log \text{volume}$
B	$\log V_x = 3{\cdot}4833 - 2{\cdot}2899 \log \text{flow} + 1{\cdot}9041 \log \text{volume}$

the volume to be 500 ml in each dog, the variance of the curve is 8·7 sec^2 in dog *A* and 4·6 sec^2 in dog *B*—a difference of more than 80 per cent. This implies that the dimensions of the circulatory system are not geometrically similar between fixed injection and sampling sites, and this seems a reasonable finding since the results were obtained from animals of widely differing physique. At the same time the relative difference in dispersion in curves obtained in a given dog from different injection sites was the same as described in Table 2.

It was found that the multiple regression function relating the variance to flow and volume, when calculated for a given injection site

from the pooled results from a number of animals, accounted less adequately for the dispersion of indicator than was the case in a well-defined system in the model. When the differences between animals were, however, taken into account, the regression function now accounted for the variation in dispersion almost as well as in the model.

The results of these experiments emphasize the quantitative importance of variation in circulatory dimensions on the dispersion of indicator substances. Additional specification of the curve is essential in the interpretation of the dispersion patterns found in a variety of circulatory disorders.

IV. The Nature of the Indicator Distribution

1. *Methods of specifying the distribution*

In the preceding discussion it was noted that the dispersion could be specified in terms of the variance of the indicator–dilution curve by a family of multiple regression equations of different intercept values. This indicates that the variance of the curve does not provide an adequate measure of the change in the symmetry of the curves between different systems. It seems very likely that if the third and fourth moments of the frequency function could be introduced as separate variables in the multiple-regression equation (equation (6)), then one multiple-regression function could account for all the variation in variance between separate systems. This has so far not been carried out but seems the best solution of the problem of specification of the curves though this method poses a number of problems owing to the incomplete nature of the primary indicator curve. This method is essentially an arithmetical process of improving the fit of the curve, but by itself provides little information about the underlying hydraulic processes. The same comment can be made in relation to the ingenious mathematical models which have been devised in an attempt to apply various frequency functions to the indicator dilution curve (Stephenson, 1948; Sheppard, 1954; Stow and Hetzel, 1954). This work has shown that certain well known distributions such as the Poisson distribution, or the log-normal distribution can be fitted to the indicator dilution curve by appropriate choice of constants. Dow (1956) has pointed out that no definite meaning has been ascribed to these constants in terms of the hydraulic factors involved in the dispersion of indicator. For this reason these results are not very amenable to practical application.

The fact that the downslope of the curves was exponential suggested to Kinsman *et al.* (1929) that the curve was basically related to the washout of a homogenously mixed volume in a flowing system. It is

well known that the exponential downslope only becomes clearly established some time after the peak of the curve has been reached, and while there is little doubt that the method of extrapolating the downslope provides an adequate estimate of the area of the curve, it does not follow that one is bound to regard the circulatory system as a uniformly mixed single compartment.

The remainder of this section presents an analysis of the change in symmetry of the curve by the use of the probit transformation. The results of this analysis indicate the nature of some of the underlying hydraulic mechanisms influencing the dispersion of indicator.

2. *The use of the probit transformation*

In the preceding discussion the indicator dilution curve has been considered as a frequency function. By integrating the curve, we obtain a cumulative frequency function which is usually termed a distribution function (Kendall and Stuart, 1958). Figure 9(a) shows the distribution function of the indicator–dilution curve previously considered in Fig. 1(a). The ordinate represents the fraction of the total amount of indicator that has passed the sampling point. The original curve has been extrapolated to the arbitrary limit indicated by the arrows in Fig. 1(a). This curve has a sigmoid shape but is not a Gaussian distribution function as may be demonstrated by using the probit transformation (Finney, 1947). This transforms a Gaussian distribution function into a straight line.

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In Fig. 9(b) the percentages of the area have been converted to probits. It is evident that this cannot be fitted by a single straight line, but that instead two straight lines can be fitted to this function. This suggests that the indicator–dilution curve could be considered as consisting of two overlapping, approximately Gaussian, components. For the present, however, the use of this function as an estimate of the degree of asymmetry of the indicator–dilution curve will be considered.

Two probit-regression lines are fitted to the points; the first probit-regression line is calculated from all points between A and B, while the points between C and D are used for calculation of the second probit-regression line. The interaction between B and C is ignored. The calculated regression lines are given in Table 4.

The ratio of the slopes of the probit-regression lines varies inversely as the standard deviation of the two components of the distribution:

$$b_1/b_2 = s_2/s_1 \quad . \quad . \quad . \quad (7)$$

where b_1 and b_2 are the regression coefficients of the first and second regression line, and s_1 and s_2 are the estimated standard deviations of

the two components of the distribution.

TABLE 4

Example of Fitting Two Probit Regression Lines to the Function Shown in Fig. 9(b)

	Item	*Value*
First probit regression line (*A–B*)	Regression equation	$Y_1 = 2{\cdot}984 + 0{\cdot}5516\ (t - t_a)$
	Standard deviation ($1/b_1$)	$1/0{\cdot}5516 = 1{\cdot}813$ sec
Second probit regression line (*C–D*)	Regression equation	$Y_2 = 4{\cdot}475 + 0{\cdot}2342\ (t - t_a)$
	Standard deviation ($1/b_2$)	$1/0{\cdot}2342 = 4{\cdot}268$ sec

t = time in seconds. Y = probit.
t_a = appearance time. $b_1/b_2 = 2{\cdot}36$.

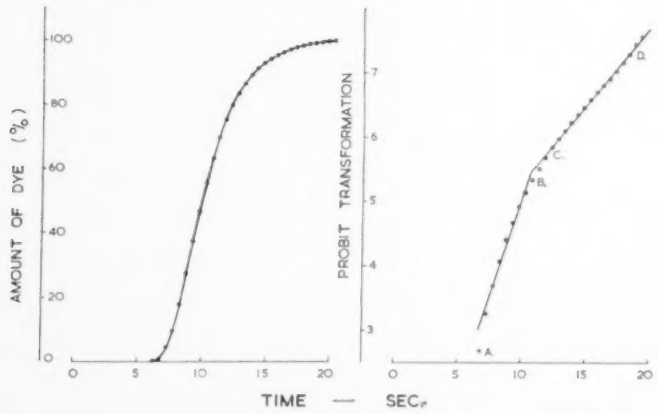

FIG. 9. (a) The distribution function of the indicator dilution curve shown in Fig. 1.
(b) Probit transformation of the distribution function.

It has been found that the point of intersection of the two probit lines falls generally at about the same ordinate value (Fig. 10 (a), (b); Fig. 20). The ration b_1/b_2 thus provides a measure of the asymmetry of the curve, since as the value b_1/b_2 increases from 1·0 the curve becomes more asymmetrical; when the ratio b_1/b_2 approaches 1·0 the whole dilution curve becomes Gaussian.

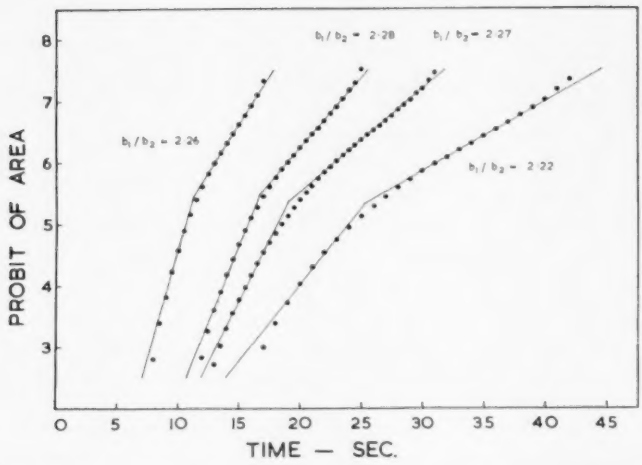

FIG. 10. (a) Probit transformation applied to indicator dilution curves obtained in the model in a system of constant path length. Flow and volume in curves from left to right: 3·9 l./min, 750 ml; 2·7 l./min, 750 ml; 2·7 l./min, 860 ml; 2·7 l./min, 1200 ml.

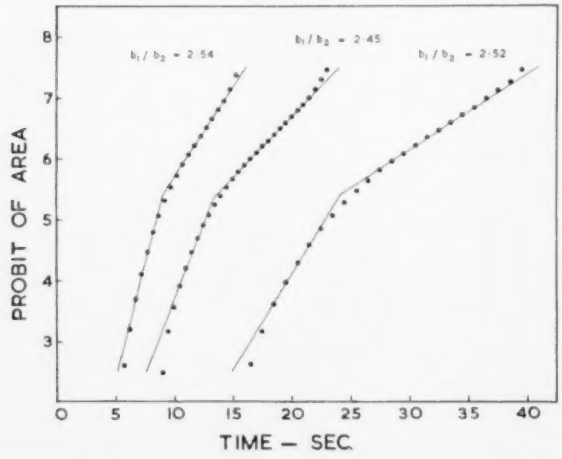

FIG. 10. (b) Probit transformation applied to curves obtained in a system of shorter path length. Flow and volume in curves from left to right 3·9 l./min, 620 ml; 2·7 l./min, 600 ml; 1·3 l./min, 560 ml.

It should be noted that in applying the probit transformation more weight is attached to points near the centre of the distribution. Points at the extremities may therefore deviate more from the probit regression line than if least-squares fitting were applied (cf. Fig. 10). The method of weighting is described fully by Finney.

3. *Factors influencing the ratio b_1/b_2 in the model*

The significance of the two components of the indicator–dilution curve can be best appreciated by referring to the results of Taylor (1954a) concerning the dispersion of indicator substances in turbulent fluids. Taylor suggested that the indicator is gradually transferred from the boundary region of the tube to the central part of the fluid, so that as the distance between injection and sampling point increases the dispersion of all the indicator approaches the Gaussian form. When sampling at a shorter distance from the injection site during constant-flow conditions a given degree of asymmetry of the curve would be expected. In the circulation the concept of a rapidly moving, well-mixed component of blood, and a more slowly moving "boundary" component has quite a lot of reality, though the division between the two components is somewhat indefinite. The rapidly moving component would consist of fluid moving near the centre of vessels and those parts of the heart chambers where mixing is good and clearance of indicator rapid. The "boundary" component would include regions where the circulatory mixing and consequent clearance of indicator is relatively slow (e.g. the regions near the walls of vessels and cardiac chambers).

In the circulation model (Fig. 2) one would expect that within any system of fixed path length and approximately constant type of flow, the relative dispersion of indicator in the two components of the dilution curve should remain constant. This can be seen in Fig. 10(a) where b_1/b_2 remain approximately constant, although the flow and volume varied widely in different curves. Figure 10(b) shows the probit functions of curves obtained in a system of shorter path length. The ratio b_1/b_2 is again approximately constant, but its value differs from the value observed in the curves from the longer system.

Figure 11 shows the average values of b_1/b_2 observed in four systems of different path length. The ratio is maximal when the path length is short and diminishes as the path length increases in approximately linear fashion. This indicates that the degree of asymmetry is reduced with longer distance between injection and sampling sites. Extrapolation of the relationship in Fig. 11 shows that the ratio tends towards 1·0 at a distance of 365 cm. The approximate average diameter of the system was about 2·5 cm, and the results are thus in reasonable agreement with those of Taylor who observed a Gaussian distribution at 100

tube diameters from the injection site under conditions of more marked turbulence.

Thorburn *et al.* found in their model that in a system of constant path length and constant flow and volume the indicator curve was significantly less dispersed at fast pump rates than at slow pump rates. The difference is only small (Fig. 12), and a large number of observations

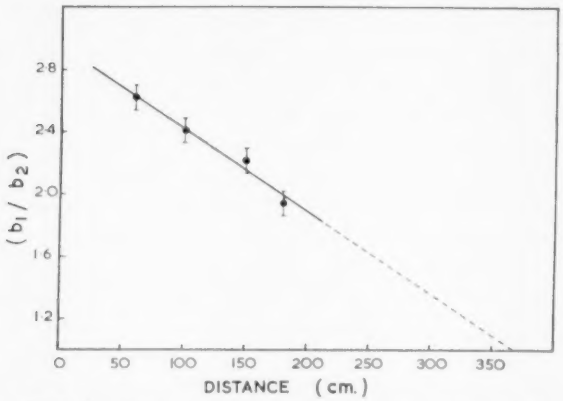

FIG. 11. Relationship of b_1/b_2 to the distance between injection and sampling sites in four systems in the circulation model. Each point is the mean of eight curves and the line on either side of each point is twice the standard error of the mean.

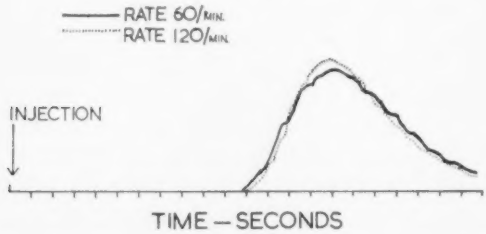

FIG. 12. Effect of variation in the pump rate on the contour of the indicator dilution curve in the circulation model. The flow and volume were maintained constant.

were required to establish its significance. At high pump rates the indicator distribution thus becomes somewhat more symmetrical. Hale *et al.* (1955) have shown that in a system with pulsatile flow the flow conditions become more unstable at high pulse frequencies. The results thus indicate that with an increase in "effective Reynolds number" (Hale *et al.*, 1955) the dispersion in the "boundary" zone becomes reduced.

4. *Factors influencing the ratio b_1/b_2 in the mammalian circulation*

The dimensional differences between individual animals and between different injection sites have been expressed by changes in the intercept value of the multiple-regression equations relating the variance to flow and volume. Figure 13 shows the relationship of b_1/b_2 to the intercept values of regression equations calculated for individual dogs in three different injection sites. The values of b_1/b_2 were calculated from the data of Korner *et al.* (1960). The ratio b_1/b_2 is clearly related to the varying intercept values and is thus of use in the additional specification of the indicator dilution curve.

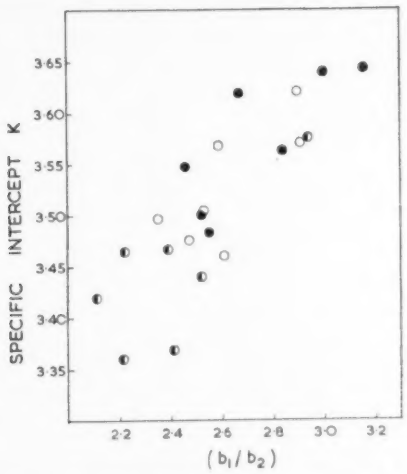

FIG. 13. Relationship of b_1/b_2 to specific intercept values of regression equations calculated for individual dogs. Each point is the mean specific intercept for each dog in curves obtained for a given injection site. ● Pulmonary artery injection. ○ Right ventricle injection. ◐ Superior vena cava injection.

The average value of b_1/b_2 in curves obtained after pulmonary artery injection was 2·73 $\pm$0·065; after right ventricular injection it was 2·62 $\pm$0·063; after superior vena caval injection it was 2·41 $\pm$0·071. This indicates that the curves became more symmetrical as the distance between injection and sampling site increased, and Dow (1954) has shown that the asymmetry is greatest in the short path length system after left ventricular injection. Rapaport *et al.* found that the degree of asymmetry is slightly less in curves determined by means of a red-cell label than in simultaneously determined curves using a plasma label. Axial movement of red cells in the smaller pulmonary vessels can account for the difference.

This increase in symmetry with increased distance can, however, become distorted by several factors. Indicator–dilution curves are often markedly asymmetrical following peripheral arm-vein injections (Hetzel *et al.*, 1954). Helps and McDonald (1954) have shown that flow in peripheral veins is strictly laminar, so that dye injected in a peripheral vein becomes dispersed in its passage to the central veins. Consequently, the arterial curve will also show more marked dispersion. Another example of the effect of non-uniform flow conditions near the injection site on the ultimate distribution of indicator was observed after right atrial injection (Edwards and Korner, 1958). In a high

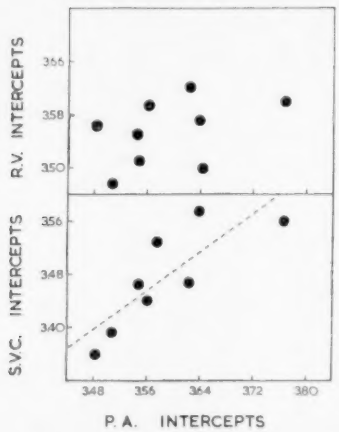

FIG. 14. Relation of specific pulmonary artery equation intercept value, to specific right ventricle equation intercept (upper graph), and specific superior vena cava equation intercept (lower graph). Each point represents the mean of from two to four intercepts calculated for each dog in each site. (From Korner *et al.*, 1960.)

proportion of curves obtained after injection in the latter site the degree of asymmetry was more marked than when indicator was injected into the inferior vena cava only a few centimetres upstream. Because the right atrium is the junction of the paths from superior and inferior vena cava the injected indicator will be initially dispersed over a larger volume than if injection into the great veins was carried out; the increased asymmetry of the curve is probably due to non-uniformity of clearance from the wall of the atrium. The same effect can be seen in the relationship between the intercept values of the regression equations from different injection sites compared in individual dogs (Fig. 14). When the intercept values from the pulmonary artery equations are plotted against those of the superior vena cava equations,

the latter values are always lower than the former and are reduced in proportion. When the pulmonary artery intercepts are compared with the corresponding right ventricular intercepts, the latter are significantly below the former, but the degree of reduction is much more variable (Korner *et al.*, 1960).

These examples indicate that the change in symmetry of the curve with different distances between injection and sampling sites is most obvious when the indicator is injected into central vessels with relatively comparable velocity distributions. Injection of indicator into regions with an obvious "boundary" component (e.g. a large cardiac chamber) tends to accentuate the asymmetry of the final curve. It is obvious that non-uniform clearance of indicator will affect the curve more when the cause of non-uniformity is near the injection site, than when the mixing is non-uniform at points downstream, since this leads to the greatest temporal separation of the two components of the indicator distribution (cf. Taylor, 1953).

The ratio b_1/b_2 can be introduced in the multiple-regression function to specify the variance for a given flow and volume with greater accuracy; when this is done the residual mean square is reduced approximately by half (Table 5). Still greater accuracy could probably be achieved by more elaborate mathematical analysis.

TABLE 5

A. Relationship of Variance, to Flow and Volume in fifty-five Dye Dilution Curves obtained in nine Normal Dogs after Pulmonary Artery, Right Ventricular and Superior Vena Caval Injection

B. Relationship of Variance, to Flow, Volume and b_1/b_2 in the above Data

A	*Regression equation*	$\log V_x = 3{\cdot}6992 - 2{\cdot}4318 \log \text{flow} + 2.0200 \log \text{volume}$
	*Residual mean square of log V_x**	0·009131
B	*Regression equation*	$\log V_x = 3{\cdot}0965 - 2{\cdot}3132 \log \text{flow} + 1{\cdot}9051 \log \text{volume} + 0{\cdot}1950\ b_1/b_2$
	*Residual mean square of log V_x**	0·005421

* Significantly different from one another.

5. *Mixing of indicator in the mammalian circulation*

All haemodynamic studies support the concept that mixing of indicator is not a uniform process in any part of the circulatory system. Helps and McDonald (1954) have shown that considerable vortex formation is observed in the great veins during the various phases of the cardiac and respiratory cycle, but this does not include the region near the wall of the vessel. Similar instability of the flow has been observed near the centre of the stream of the rabbit aorta during the systolic ejection phase of the cardiac cycle (McDonald, 1952). In the ventricle mixing is not completely uniform: differences in blood–gas composition in the aorta and pulmonary artery were observed by Callahan *et al.* (1955) in patients with an anatomically common ventricle, where the diagnosis was confirmed at necropsy. Additional evidence on this point will be considered in relation to the quantitative estimation of valvular incompetence and various types of congenital shunts.

The above findings support the view that the indicator–dilution curve can be regarded as the sum of two components resulting from non-uniform mixing in the circulation. The demonstration that the curve tends to the Gaussian form with increased distance between injection and sampling sites and an increase in the Reynolds number are also in agreement with this idea. It will be seen that this concept is of value in the practical application of the indicator-dilution method to the quantitative estimation of many types of circulatory disorders.

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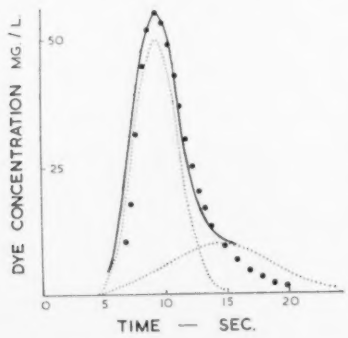

FIG. 15. The dotted curves represent the two hypothetical Gaussian components of the dye-dilution curve. Curves are calculated from the mean time of each component, and the standard deviation. The heavy-lined curve represents the resultant hypothetical dilution curve. The plotted points represent the actual concentration values of the curve shown in Fig. 1(a).

An approximate calculation of the two Gaussian components is shown in Fig. 15. Exact definition of the means of each component is difficult owing to the overlap, but on the whole there is a reasonable degree of agreement between the hypothetical curve and the original points.

V. ESTIMATION OF FLOW and "SPECIFIC" VOLUMES FROM PORTION OF THE INDICATOR DILUTION CURVE

1. *Use of the first part of the curve as an estimate of total area*

In patients with large central blood volumes (e.g. patients with mitral stenosis and left atrial enlargement), recirculation of indicator may commence before the downslope of the curve has been clearly defined. This was more common when peripheral arm-vein injections were used, but with the increasing use of central injection sites, this type of curve is encountered less frequently. In patients with central left-to-right shunts, however, the indicator–dilution curves have a very irregular downslope owing to the complex nature of the pathways traversed by the indicator. Under these conditions extrapolation of the downslope by the Hamilton method is not possible, and the area of the curve cannot thus be directly determined. In view of the well-defined nature of the first part of the curve, empirical formulae have been developed to predict the whole area from this part of the dilution curve.

The first prediction formula of this type was due to Dow (1954); he was well aware of the limitation of this approach because of the variation in the degree of asymmetry of the curves and attempted to allow for this by means of a number of time component ratios. Comparison of estimates of flow obtained by means of his formula with the results of the conventional calculation (equation (1)) gave agreement to ± 25 per cent in about two-thirds of cases, with greater degrees of divergence in the remainder. This suggests that the formula does not completely allow for the dimensional variations between individuals.

A formula which has been more commonly employed, and which gives results essentially similar to the Dow formula was developed by the group of workers at the Mayo Clinic. Warner and Wood (1952) simplified the calculation of the area of the first part of the curve by considering it as a right-angled triangle, termed the forward triangle. One side of the right-angled triangle is the build-up time (the time between the first appearance of the indicator and the peak concentration) and the other side is the peak concentration. The relationship of the area of the forward triangle to the total curve area was examined by Hetzel *et al.* (1958) in curves obtained from human subjects, using a number of injection sites. If this part of the curve were always a

fixed proportion of the total area, then the relationship could be expressed by

$$k = \tfrac{1}{2}\, t_b\, C_p/A \quad . \quad . \quad . \quad (8)$$

where k = proportionality constant;
t_b = build-up time;
C_p = peak concentration;
A = area of the whole curve.

The flow can then be estimated from the relationship

$$F = 60\, I\, k/\tfrac{1}{2}\, t_b\, C_p \quad . \quad . \quad . \quad (9)$$

Hetzel *et al.* found that the mean value of the proportionality constant varied from 0·38 in curves obtained after pulmonary artery injection, to 0·32 in curves obtained after peripheral vein injections. There was a range of variation of some 70 per cent in the value of this constant in curves from a given injection site. This is undoubtedly related to the heterogeneity in the indicator distribution between different individuals discussed in the preceding section, and the flow can thus be estimated only approximately by the formula. The smaller value of the proportionality constant after peripheral vein injection is probably due to the greater contribution made by the "boundary" component owing to the initially uneven velocity distribution. The fact that the formula gives approximate estimates of flow is explained by the relatively minor contribution of the "boundary" component to the area of the whole curve. It should be noted that the forward-triangle method is a simple way of specifying the first Gaussian component (see Fig. 15). It will be seen that as applied to the estimation of central left-to-right shunts, the effect of dimensional variation between individuals is effectively minimized, and under these conditions equation (9) gives more accurate estimates of flow.

2. *Estimation of pulmonary and ventricular residual volumes from analysis of the downslope*

Newman *et al.* (1951), constructed a circulation model in which fluid passed through a number of bottles of varying size, connected in series. By means of various stirring devices uniform and practically instantaneous mixing of indicator took place as it passed through each compartment. The largest compartment imparted its own characteristic downslope to the final dilution curve; Newman *et al.* concluded that the volume of the largest compartment could be estimated from the relation "V" = s/F, where "V" is the volume of the largest compart-

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ment, F the flow and s the downslope of the curve. They considered that this concept, when applied to the circulation would give estimates of the volume of the pulmonary vascular bed. This theory has been criticized on a number of occasions (Meier and Zierler, 1954; Sheppard, 1954; Dow, 1956) and the model bears little relation to the actual mixing conditions found in the circulation. From the discussion of Section IV it is evident that the downslope lies in the region of maximum heterogeneity of the indicator distribution (see Fig. 15); it seems unlikely that analysis of the downslope will provide information about any specific anatomical volume, except in the special case when the distance between injection and sampling site is small, and the "boundary" region has definite anatomical significance.

This form of analysis thus has application in the estimation of residual ventricular volume, when indicator is injected into the ventricle and sampled from the aorta or pulmonary artery (Bing *et al.*, 1951; Newman *et al.*, 1951; Holt, 1956). In this case the indicator distributed in the stroke volume represents the first component of the distribution, while the indicator distributed in the residual volume represents the "boundary" component. A dilution curve recorded under these conditions is markedly asymmetrical, and the peak concentration value is reached in the first systole after injection (Holt, 1956). It seems that analysis of the downslope may provide information about the extent of the "boundary" component, though the results of Holt indicate that there are considerable difficulties associated with the estimation of flow from the area of the curve when the distance between injection and sampling points is small. Experiments in dogs and in models have provided estimates of a reasonable order of magnitude, but no rigorous checks of the formulae developed have so far been carried out.

VI. Indicator Dilution Curves in Valvular Incompetence

1. *Qualitative changes in the curves*

In incompetence of the semilunar valves the volume ejected by the ventricle during systole includes the forward stroke volume, as well as the regurgitant stroke volume. The latter subsequently returns to the ventricle in diastole. This flow pattern is shown in Fig. 16 in dogs with variable amounts of pulmonary valve incompetence. When the atrioventricular valves are incompetent, part of the systolic stroke volume is ejected forward by way of the semilunar valves, and part of it regurgitates into the atrium by way of the incompetent tricuspid or mitral valves (Müller and Shillingford, 1955). When indicator is injected at or behind the zone of incompetence (i.e. into the ventricle or atrium in incompetence of the semilunar valves, and into the atrium or great veins in incompetence of the atrioventricular valves), its rate

of movement is thus increased in one phase of the cardiac cycle, and retarded in the other. In incompetence of the semilunar valves movement of indicator is accelerated during systole and retarded in diastole, since some particles regurgitate into the ventricle during the latter

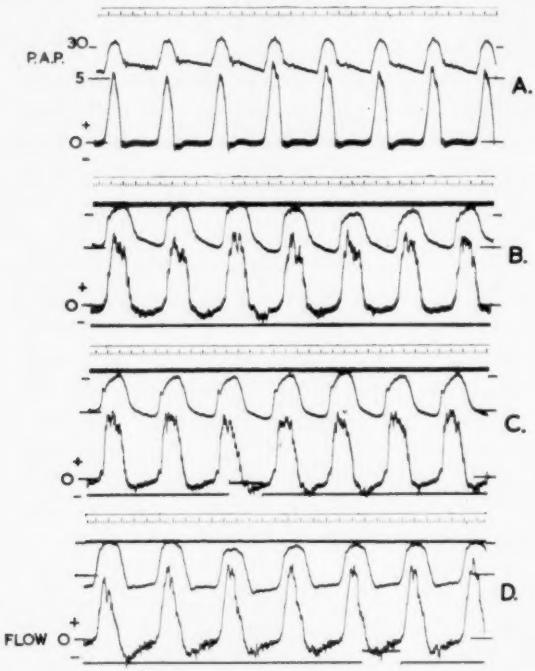

FIG. 16. Records of pressure and flow in the main pulmonary artery of dogs with varying degrees of pulmonary valve incompetence. Flow was recorded with a bristle flowmeter and is proportional to the square root of the deflexion. The sensitivity of the flowmeter differed slightly in each record. Each record from top to bottom: time marker (large division) 0·1 sec; pressure in pulmonary artery; flow; event marker. The line just under the time marker in records *B* to *D* is the baseline of the simultaneously recorded indicator dilution curve. (From Korner *et al.*, 1960.)

phase of the cycle. In mitral or tricuspid incompetence, retardation of indicator particles occurs in systole, since some particles move back into the atrium; the rate of movement of indicator is accelerated in diastole since the regurgitant flow from the previous systole is augmented by the normal venous return.

It is thus clear that the dispersion of indicator recorded at a distant sampling site is increased by valvular incompetence (Kopelman and

Lee, 1951; Korner and Shillingford, 1955; Conn *et al.*, 1957; Woodward *et al.*, 1957a; Korner *et al.*, 1959). This is not specific to valvular incompetence and occurs in central left-to-right shunts; in fact valvular incompetence can be regarded as a very limited type of left-to-right shunt. The curves in valvular incompetence have a well-defined downslope and valid estimates of forward flow and volume can be obtained from the area of the curve and the mean circulation time (Korner and Shillingford, 1955; Korner *et al.*, 1959).

The upper set of records in Fig. 17 were obtained in the model of Thorburn *et al.* The inflow valve of the pump was competent and the outflow valve could be either competent or incompetent; under both

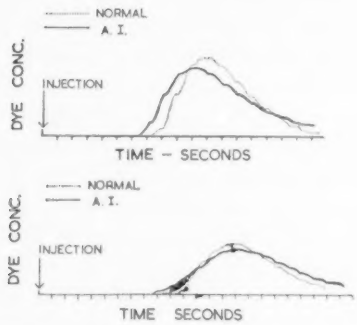

FIG. 17. *Upper curves:* Records obtained in the model with normal valves and during "aortic" incompetence (A.I.) following dye injection close behind the "mitral" valve. Forward flow 2·7 l./min, volume 580 ml in both curves. Actual backflow during valve incompetence 1·4 l./min.

Lower curves: Records obtained in the model with valves normal and during "aortic" incompetence following dye injection far upstream from the mitral valve. The volume between injection site and zone of incompetence was approximately 500 ml. Forward flow 3·4 l./min, volume 1080 ml in both curves. Actual backflow during valve incompetence was 1·7 l./min. (From Korner *et al.*, 1959.)

conditions forward flow and volume were kept constant. Indicator was injected close behind the mitral valve (point *G*, Fig. 2). The presence of incompetence results in an earlier appearance of indicator at the distant sampling site; the peak concentration is lower and the peak value is earlier in incompetence; the downslope is more prolonged and the dispersion of indicator is increased. Similar effects can be seen in a dog with experimental pulmonary valve incompetence (Fig. 18). The mean circulation time is the same under the above conditions, whether valve incompetence is present or not. Valvular incompetence thus

produces greater dispersion of indicator about the mean, clearly related to the characteristic pattern of double flow.

The change in contour of the curves has been used in the localization of valvular incompetence. The site of the lesion can be identified either by the use of multiple injection sites, or multiple sampling sites (Korner and Shillingford, 1955; Braunwald *et al.*, 1957a; Wright and Wood, 1957). Curves obtained from a distant sampling site have a normal contour if indicator is injected distal to the next competent valve lying downstream from the incompetent valve; curves of abnormal contour are obtained if injection is carried out at the zone of incompetence, or at points upstream from this site.

In the multiple-sampling technique, indicator is injected in the venous side of the circulation and curves of abnormal contour are recorded from points at, or downstream to the incompetent valve. Curves recorded from sites upstream to the next proximal competent valve have an essentially normal contour.

A number of qualitative tests have been used to diagnose the occurrence of valvular incompetence in the presence of valvular stenosis since the differentiation is often clinically difficult (Woodward, *et al.*, 1957a; Wood and Woodward, 1957; Warner and Toronto, 1958; Shillingford and Zoob, 1957). The simplest of these tests is shown in Fig. 19. In normal subjects, or in patients with valvular stenosis the least concentration of indicator just before recirculation (C_L) is well below the recirculation peak (C_R). In the presence of valvular incompetence this oscillation of the indicator curve is damped out and measurement of the ratio C_L/C_R affords a reasonably successful simple differentiation between valvular stenosis and incompetence (Wood and Woodward, 1957).

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2. *Quantitative estimation of valvular incompetence*

The indicator dilution method offers the only approach available at present, to the estimation of regurgitant flow in intact animals. Results obtained in humans and in dogs have shown that the order of magnitude of the backflow can exceed the net forward flow (Korner and Shillingford, 1955; Korner *et al.*, 1960). Valvular incompetence produces marked alterations in the venous pressure, regulation of the cardiac output and in the body-fluid balance (Barger *et al.*, 1952; Barger *et al.*, 1955; Müller and Shillingford, 1955; Braunwald *et al.*, 1957b; Korner and Shillingford, 1957; Welch *et al.*, 1957). The development of a technique for the quantitative estimation of valvular incompetence has thus interesting physiological applications.

Two methods have been developed to estimate the amount of regurgitant flow. In one of these, which we may call the dispersion

method, the dispersion of indicator is compared during valvular incompetence, with the dispersion in an identical system with competent valves at constant flow and volume (Korner and Shillingford, 1955, 1956; Novack and Schlant, 1958; Woodward *et al.*, 1957a; Korner *et al.*, 1959, 1960). The indicator is injected just upstream from the zone of incompetence and the time–concentration curve recorded from a distal arterial sampling site. The method requires accurate specification of the system under study.

The second method may be termed the multiple-sampling method. Indicator is injected downstream immediately adjacent to the incompetent valve, and curves are recorded at either side of the incompetent valve. The amount of backflow is estimated from the relative areas of the two curves (Woodward *et al.*, 1957b; Bajec *et al.*, 1958; Lacy *et al.*, 1959).

The success of either method depends largely on how far the assumptions are made consistent with the facts of normal indicator dispersion in the mammalian circulation. Both methods have given good results in models, but the difficulties of this type of approach has been well expressed by Dow, when he writes: "Hydraulic models, in general, demonstrate only how close one can build to theoretical assumptions, not at all how close the theory or the model approximates the cardiovascular system".

3. *Dispersion method*

3.1. *Theoretical considerations.* Since under the conditions of the experiments shown in Figs. 17 and 18 the mean circulation time remains unchanged, the variance of the curve obtained during valvular incompetence can be regarded as having a component ascribable to the normal forward flow and volume, and a component due to backflow:

$$V_x \textit{ observed} = V_x(F;V) + V_x(B;V) \quad . \quad . \quad . \quad (10)$$

where V_x observed = calculated variance of the curve obtained during valvular incompetence;

$V_x(F;V)$ = expected variance for the same forward flow and volume, with the valves competent using the same injection and sampling sites;

$V_x(B;V)$ = component of variance due to backflow.

The amount of backflow is given by the relationship:

$$V_x \textit{ observed}/V_x(F;V) = \text{total flow/forward flow} \quad . \quad . \quad . \quad (11)$$

where total flow = forward flow + backflow.

The forward flow and V_x *observed* can be calculated from the curves recorded during valve incompetence by the usual methods. The

expected variance $V_x(F;V)$ must be calculated from multiple-regression equations relating the variance of the curve to flow (i.e. forward flow) and volume. It follows from the discussion in Sections III and IV that this value must be calculated for an identical system.

The following assumptions are implicit in equations (10) and (11):

(1) The component of variance due to backflow adds to the component of variance for the same forward flow and volume.

(2) The experimental conditions must be such that the mean circulation time remains in fact unaltered by the presence of valvular incompetence, i.e. the increased dispersion must be independent of the volume between injection and sampling sites.

(3) The component of variance due to backflow is directly proportional to the true backflow through the incompetent valve.

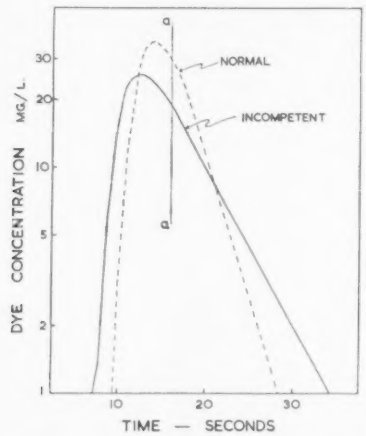

FIG. 18. Replotted dye dilution curves obtained from one dog before and after the production of pulmonary valve incompetence. Dye was injected into the right ventricle on both occasions.

Normal curve: forward flow = 1·84 l./min; volume = 488 ml; t. = 15·9 sec; V_x = 12·06 sec².

Pulmonary incompetence: forward flow = 1·89 l./min; volume = 510 ml; $\bar{t}$ = 16·2 sec: V_x = 27·5 sec².

Flowmeter results: total flow/forward flow 2·33. (From Korner *et al.*, 1960.)

If the indicator is injected just behind the zone of incompetence, any superimposed distribution due to valve incompetence must be represented in all components of the dilution curve. This is demonstrated in Fig. 20 where the probit transformation has been applied to the curve shown in Fig. 18. The dispersion of indicator is increased to the

same extent in both components of the distribution, since the ratio b_1/b_2 is approximately the same before and after the production of valvular incompetence. This suggests that the dimensions of the system have remained unchanged. The mean time of the first component of the distribution decreases, while the mean time of the second component increases, so that the mean circulation time remains unchanged.

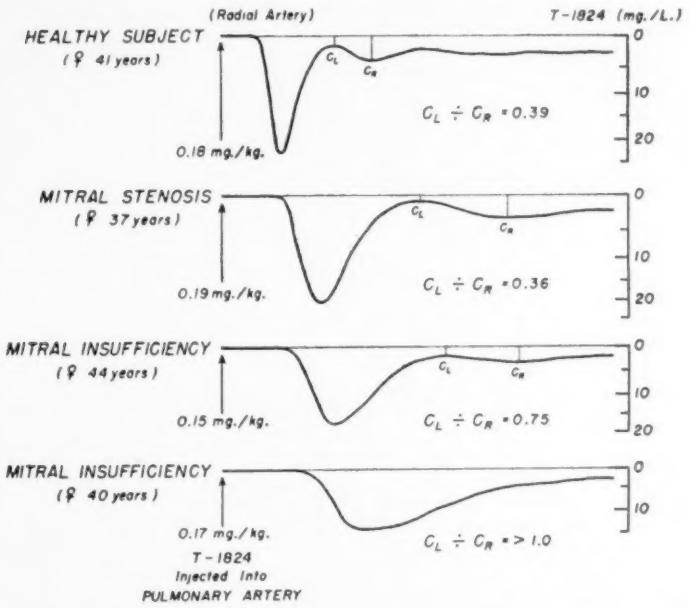

FIG. 19. Relationship of least concentration value (C_L) to recirculation peak (C_R) in normal subjects, and in patients with valvular stenosis and incompetence. (From Wood and Woodward, 1957.)

The experimental conditions required to satisfy assumption (2) must be such, that *all* indicator particles must have the same chance of being either accelerated or retarded when passing through the zone of incompetence (Korner *et al.*, 1959). The necessary experimental conditions can be illustrated conveniently in the model.

When indicator is injected just behind the "mitral" valve in "aortic" incompetence (position G, Fig. 2) the volume of the system is essentially fixed whether valve incompetence is present or not. All indicator particles must pass through the zone of double flow, and each has the same chance of being accelerated through this region, or of regurgitating back into the "ventricle". Fig. 21 (a) shows that the mean circulation

time remains approximately constant for a given forward flow when the valves are normal, or when incompetence is present. The increased dispersion in incompetence, shown by the reduction in appearance time for a given mean circulation time in Fig. 21(b), is thus independent of volume. The same result was obtained in "mitral" incompetence, using an elastic "atrium", when the injection site was in the proximal part of the "atrium".

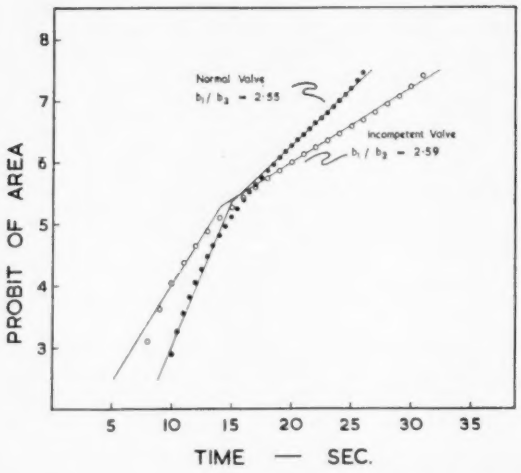

FIG. 20. Probit transformation applied to the curves shown in Fig. 18, obtained in a dog before and after the production of pulmonary valve incompetence. • Valves normal. ○ Pulmonary valve incompetence.

When, during "mitral" incompetence, the indicator was injected into the "ventricle" the estimated volume was larger than when the valves were normal. In the first diastole after injection there is little acceleration of injected indicator particles; in the following systole some particles will be ejected into the arterial system, some remain in the "ventricle", and some regurgitate into the "atrium". Only the particles in the latter group will have the same chance of being accelerated or retarded through the zone of double flow during the next cycle. After "ventricular" injection in "mitral" incompetence the estimated backflow from equation (11) is always below the true backflow (Korner *et al.*, 1959). In dogs with experimental incompetence, injection behind the zone of incompetence is also essential to achieve the required distribution of indicator (Korner *et al.*, 1960). Another condition in which maldistribution of indicator occurred in the model was in "mitral" incompetence when the "atrium" was a rigid tube (Hoffman

and Rowe, 1959; Korner *et al.*, 1959); this is not of practical significance in relation to any condition found in the mammalian circulation.

In order to satisfy assumption (3) injection must be carried out *close* behind the zone of incompetence. Indicator then enters this region as a compact undispersed mass and can be partitioned in the proportion of forward flow to backflow. Results obtained in the model in "mitral" or "aortic" incompetence showed good agreement between the estimated backflow using equation (11) and the observed backflow (Fig. 22). In dogs with experimental pulmonary incompetence the estimated backflows were compared with simultaneously determined estimates

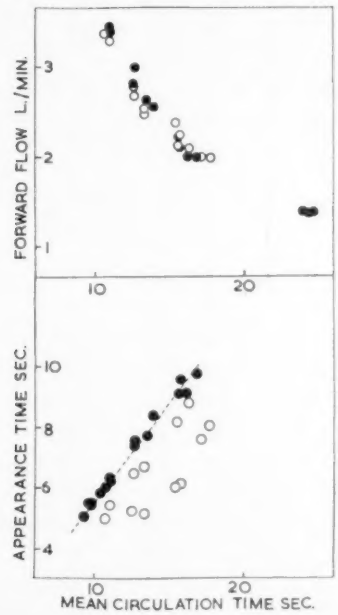

FIG. 21. (a) Relationship of the mean circulation time to the forward flow in the circulation model, with the valves normal, and during "aortic" incompetence. Injection close behind the zone of incompetence. (b) Relationship of mean circulation time and appearance time, with valves normal and during "aortic" incompetence. • Valves normal. ○ "Aortic" incompetence.

obtained by means of a bristle flowmeter (Korner *et al.*, 1960). The intercept value of the multiple-regression equations used in the calculation of $V_x(F;V)$ was determined in each individual animal from normal curves obtained before the production of valvular incompetence ("specific" regression equations). The scatter of individual estimates of backflow by the dye method was about ± 30 per cent

(s.d.); this large scatter was probably due to beat-to-beat variation in the forward flow and backflow (cf. Fig. 16). The errors introduced in this way were random in nature, since the scatter was greatly reduced (±14 per cent s.d.) by comparing the mean of three to four successive dye estimates, with the mean of simultaneous flowmeter estimates, obtained while the animal was in a steady state (Fig. 23(a), upper panel). The accuracy of the average of from three to four successive estimates of backflow thus approached the accuracy of a single estimate of forward flow by the direct Fick or indicator–dilution method. These results indicate that the method can be applied in the mammalian circulation.

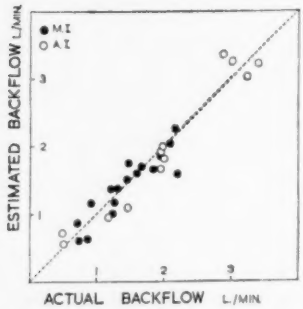

FIG. 22. Relationship of estimated backflow by the dispersion method, to actual backflow, during "aortic" or "mitral" incompetence in the model, following dye injection close behind the zone of incompetence. (From Korner *et al.*, 1959.)

When the injection site is moved far upstream from the zone of incompetence, estimates of backflow can no longer be obtained by the use of equation (11), since assumption (3) is no longer satisfied. In experiments carried out by Hoffman and Rowe (1959), injection was carried out through a large volume situated upstream from the zone of incompetence. They observed little change in dispersion in valve incompetence and concluded that the dispersion method was unreliable in the estimation of backflow. Korner *et al.* (1959) confirmed the observations of Hoffman and Rowe, and analysed the mechanism of this effect (Fig. 17). When the injection site is just behind the zone of incompetence there is a much greater difference in dispersion between a curve obtained during incompetence and one obtained with normal valves, than when the volume between injection site and zone of incompetence is large. This is due to the fact that in the latter case the indicator is already widely dispersed before its arrival in the zone of incompetence; excess dispersion can only occur along the edges of the oncoming distribution, and in the centre of the distribution indicator particles merely exchange places with one another. On the other hand, when the indicator enters the zone of incompetence as a fairly compact mass, maximum dispersion of particles from the mean of the distribution is achieved. Korner *et al.* showed that the degree of underestimation

of backflow obtained by using equation (11), was directly related to the amount of initial dispersion acquired by the indicator between the injection site and the zone of incompetence. The true backflow could be estimated from the relationship

$$(V_x \textit{ observed} + d)/V_x(F;V) = \text{total flow/forward flow} \quad . \ . \ . \quad (12)$$

where d = variance due to dispersion between injection site and the zone of incompetence.

Equation (12) has no practical significance, and merely explains an apparent anomaly in the dispersion method. The findings demonstrate that the correct initial partitioning of the indicator is an essential requirement of the dispersion method, and that this can be obtained by suitable choice of injection site.

A somewhat similar effect was observed in the model when both the inflow and outflow valves were incompetent. It was found that when the indicator was injected in the atrium the estimate of backflow was always below the combined backflow through both valves. Under these conditions the indicator is probably widely dispersed by the incompetence of the inflow valve, so that the additional effect due to incompetence of the outflow valve is minimized. The dispersion method can thus be readily applied only to cases of incompetence of a single valve.

When indicator is injected remote from the zone of incompetence, the system has a region of increased oscillation due to the presence of backflow. The relatively slight increase in dispersion under these conditions makes it unlikely that the phasic backflow which is observed in part of the arterial system plays a significant role in the dispersion of indicator in the normal circulation.

3.2. *Practical application of the dispersion method in the mammalian circulation.* The results obtained in dogs showed that if the expected variance $V_x(F;V)$ was calculated from "specific" regression equations reasonable estimates of backflow were obtained. In the clinical application of the method "specific" regression equations are not available for a given patient, and other means must be used to calculate the value of $V_x(F;V)$.

Some of the difficulties can be illustrated in the lower panel of Fig. 23(a), where $V_x(F;V)$ was estimated from "general" regression equations. These were obtained from the pooled data of all dogs for each injection site (Table 2). The estimates obtained when calculating $V_x(F;V)$ in this way were very poor, and the method barely served as a qualitative guide to the amount of backflow. The discrepancy in results obtained by the use of different estimates of $V_x(F;V)$ are obviously due to the normal variation in circulatory dimensions between different animals. The quantitative effect of this variation has

only been appreciated very recently and accounts for the disappointing clinical results obtained by calculating $V_x(F;V)$ from "general" regression equations (Novack and Schlant, 1958; Shillingford and Zoob, 1957; Woodward *et al.*, 1957a).

The main difficulty in the practical application of the dispersion method thus lies in the estimation of the expected variance, with due

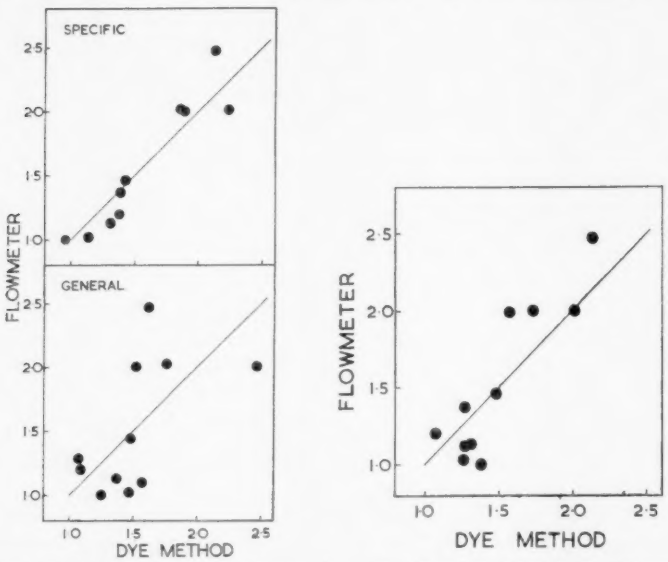

FIG. 23. (a) *Upper panel:* Comparison of estimates of total flow/forward flow by indicator–dilution (dispersion) method and by the bristle flowmeter in dogs with pulmonary valve incompetence. Estimates of $V_x(F;V)$ were obtained from specific regression equations obtained for each individual dog. Each point is the mean of from three to four successive simultaneous comparisons by the two methods. The line is the line of identity for the two methods of estimation. *Lower panel:* Comparison of estimates of total flow/forward flow by the dye and flowmeter method. Estimates of $V_x(F;V)$ were obtained from general regression equations calculated from the pooled data for each injection site. Each point is the mean of from three to four successive simultaneous comparisons by the two methods. (From Korner *et al.*, 1960.)
(b) Comparison of estimates of total flow/forward flow by the dye and flowmeter method. Estimates of $V_x(F;V)$ were calculated from the triple regression function shown in Table 5.

allowance for dimensional variation between individuals. Korner *et al.* (1960) suggested a method in which this could be done; this depends on the interrelationship between specific intercept values from different injection sites (Fig. 14). By obtaining normal curves downstream from the zone of incompetence estimates of the specific intercepts behind the

zone of incompetence could be obtained and the results showed reasonable agreement with actual backflows.

An alternative method may be applied by the use of the ratio b_1/b_2 of the probit transformation of the curve. It was seen that this ratio remains approximately constant in the presence of valvular incompetence, so that information about the dimensions of the system can be obtained from the abnormal curves themselves. The expected variance for a given forward flow, volume and probit slope ratio (b_1/b_2) can then be calculated from the triple regression equation of Table 5. This calculation has been applied to the data of Korner *et al.* (1960) and is shown in Fig. 23(b). The triple regression function was determined from curves obtained from nine normal dogs that were not used in the dye-flowmeter comparisons. The results show a great improvement compared to the estimates obtained by the use of "general" regression equations, but they are not as good as the results obtained by means of the "specific" regression equations.

The latter method is not the only way in which increased information about the dimensional variation of the system can be obtained from the abnormal curves themselves. The constancy of the ratio b_1/b_2 in the presence of valvular incompetence suggests that the asymmetry of the curve is not altered under these circumstances. The calculation of the third- and fourth-order moment of the frequency function, seems the most likely arithmetical method of improving the accuracy of prediction of the regression function.

4. *Multiple sampling method*

The theoretical formulation of this method has been given recently by Lacy *et al.* (1959) and McClure *et al.* (1959). The principle of the method can be illustrated by considering a case of mitral incompetence. Indicator is injected into the left ventricle, and simultaneous dilution curves are obtained from the left atrium and the root of the aorta. If we denote

S_F = forward stroke volume;
S_B = regurgitant stroke volume;
A_a = area of atrial dilution curve;
A_v = area of ventricular dilution curve,
$\simeq$ area of aortic dilution curve;
I_a = amount of indicator entering the atrium;
I'_a = amount of indicator leaving the atrium.

Then the total amount of indicator that has entered the atrium is given by

$$I_a = S_B A_v$$

while the amount of indicator that has left the atrium equals

$$I'_a = (S_B + S_F)\, A_a$$

Since all the indicator that enters the atrium will eventually leave it, $I_a = I'_a$, and

$$\text{backflow/forward flow} = A_a/(A_v - A_a) \quad . \quad . \quad . \quad (13)$$

The explicit assumptions in the method, made by Lacy *et al.* were as follows:

(1) The concentration of indicator in the root of the aorta is representative of the concentration in the ventricle.

(2) At the beginning of ventricular ejection the indicator has been mixed in the ventricle with sufficient uniformity to ensure that its concentration in the fluid volume ejected forward is identical with its concentration in the regurgitant volume.

(3) That mixing in the atrium is uniform by the end of diastole.

The theory was tested in a circulation model; Lacy *et al.* found it necessary to use motor-driven stirring propellers in each "cardiac" chamber to obtain sufficient uniformity of mixing. Under these conditions there was good agreement between estimated and observed backflows. The authors expressed some doubts about the validity of their assumptions in the mammalian circulation.

The qualitative use of a similar method in patients with mitral incompetence, and in dogs with experimental valve lesions by Woodward *et al.* (1957b) shows that the above assumptions are unlikely to be valid in the mammalian circulation. They found that indicator failed to appear in the atrium with disappointing frequency, when valve incompetence was known for certain to be present; occasionally dye appeared when valve incompetence was known to be absent. Similar observations were made by Bajec *et al.* (1958) in patients and dogs with tricuspid incompetence. In the upper panel of Fig. 24 dye appeared in the atrium immediately after ventricular injection in a patient with tricuspid incompetence; when the catheter was moved by a small amount in the right ventricle (lower panel, Fig. 24) scarcely any dye appeared in the atrium immediately after injection. These experiments indicate that uniform mixing in the ventricle cannot be assumed with any degree of certainty, and other evidence considered in Section VII confirms this conclusion.

5. *Comparison of the dispersion method and the multiple-sampling method*

The chief sources of error in the estimates of backflow by the dispersion method in the mammalian circulation are the result of beat-to-beat

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variation in the partitioning of the indicator, and the difficulty associated with the estimation of $V_x(F;V)$. The first factor is entirely random and can be overcome by averaging the estimates obtained from a small

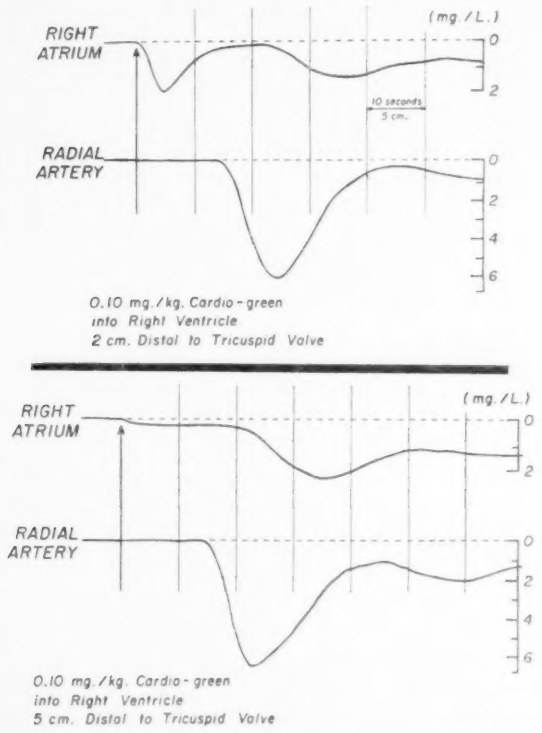

FIG. 24. Effect of variation in catheter position on the appearance of indicator in the right atrium in a patient with tricuspid incompetence. A rise in indicator concentration is shown by a deflexion below the baseline. In the upper set of curves indicator was injected into the right ventricle, with the catheter tip 2 cm distal from the tricuspid valve. Indicator appeared in the right atrium immediately after injection, before its appearance at the peripheral artery indicating the presence of tricuspid incompetence. When the catheter tip was moved slightly in the right ventricle only a very small amount of dye appeared in the right atrium immediately after injection; the major deflexion did not occur till after the arrival of indicator at the peripheral artery. The sampling site in the right atrium was the same for both sets of curves. (From Bajec *et al.*, 1958.)

number of successive curves. The problem of estimating $V_x(F;V)$ has been partially overcome and it seems likely that it will be further overcome by more elaborate statistical specification of the abnormal

curves themselves. The method makes no further assumptions about the manner of dispersion of the indicator in specific parts of the circulation. The dispersion method, though somewhat laborious, thus seems promising in the quantitative investigation of valvular incompetence in the mammalian circulation.

The multiple-sampling method is theoretically very much simpler, but it is difficult to see how its assumptions can be even approximately valid in the mammalian circulation. It seems worthwhile to consider the practical consequences of deviations from these assumptions. The first problem of the method is also common to the dispersion method, since the amount of injected indicator must be partitioned in the proportion of forward flow to backflow. This can be minimized by carrying out successive sets of curves, and from the results with the dispersion method in dogs it is clear that this problem can be easily overcome.

The question of recording representative dilution curves from the atrium and aorta is a more difficult one. The results of Holt (1956) are pertinent to this discussion; he obtained curves from the root of the aorta in *normal* dogs after left ventricular injection of indicator. He found that the accurate measurement of the area of the curve was difficult under these conditions due to irregularities in the heart rate and a variable emptying of the ventricle. This is to be expected in a situation where the injection site is very close to the sampling site (Section IV). Under these conditions the "boundary" component is of much greater significance in the estimation of the curve area, and if this component varies from moment to moment, great difficulties are to be expected in the estimation of any parameter of the curve and in extrapolating the downslope. This problem is entirely avoided by the dispersion method, since apart from errors in partitioning, the choice of a *distal* sampling site minimizes moment-to-moment irregularities in the distribution close to the inlet of the system. In the multiple sampling method in valve incompetence the problem is further accentuated since one has to consider non-uniform mixing not merely in one heart chamber, but in two. The results obtained by Wood and his colleagues at the Mayo Clinic indicate that this difficulty is very real indeed, and it seems to the writer that the problems are such that the multiple sampling method can be applied only in a qualitative manner to the estimation of valvular incompetence. It will be of interest to see the results of simultaneous comparisons of the multiple sampling method with estimates obtained by a flowmeter in the mammalian circulation. While the multiple sampling method appears relatively unsuited to the quantitative estimation of valve incompetence it will be seen in Section VII that it is the method of choice in the quantitative

estimation of central left-to-right shunts, since the main limiting condition of sufficient distance between injection and sampling site is satisfied when it is used in the latter estimation.

VII. INDICATOR DILUTION CURVES IN CONGENITAL SHUNTS

1. *Methods available for studying these defects*

The demonstration of the value of the indicator dilution method in the diagnosis and quantitative estimation of congenital shunts is almost entirely due to the work of Wood, Swan and their colleagues at the Mayo Clinic. Detailed consideration of the many uses of this method in the diagnosis of congenital heart disease is beyond the scope of this article and reference should be made to the original papers. Discussion will be limited to a few illustrative examples of the principal types of central shunts. Information concerning the location and magnitude of the various defects can also be obtained from analysis of the oxygen content of blood, obtained from various sites in the central circulation (Cournand *et al.*, 1949). This gives an independent means of checking the results of the indicator method. The latter provides more information about the nature of the abnormal circulatory pathways, and is more sensitive in detecting shunts of small magnitude (Russell *et al.*, 1958).

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2. *Veno-arterial (right-to-left) shunts*

2.1. *Qualitative changes in the curves.* An example of this type of defect is illustrated in Fig. 25, in a patient with pulmonary stenosis, ventricular septal defect and overriding aorta. The right ventricular pressure is elevated and blood is ejected from the right ventricle by one of two pathways (Swan and Wood, 1953):

(1) Into the pulmonary circulation⟶left atrium⟶left ventricle ⟶aorta⟶systemic arteries.

(2) Through the ventricular septal defect⟶left ventricle⟶ aorta⟶systemic arteries.

If indicator is injected into the pulmonary artery it travels entirely by way of pathway (1), and a curve of essentially normal contour is recorded from a peripheral sampling site. When the indicator is injected into the right ventricle, or further upstream, the curve obtained now has an appearance time that is shorter than that of the previous curve. There is a concentration hump on the upslope, which represents the passage of indicator which has travelled via pathway (2); the second peak of concentration is due to indicator which has travelled through the pulmonary circulation (Swan *et al.*, 1953). The admixture of venous blood with arterial blood in these patients results in arterial

hypoxia; the introduction of the protein-bound dye, indocyanine, with a maximum light absorption in the region of 800 mμ, by Fox *et al.* has overcome the difficulties found with T–1824 in recording these curves in the presence of a fluctuating arterial oxygen saturation.

2.2. *Quantitative estimation.* Swan *et al.* (1953) used the forward-triangle method (p. 143) in the estimation of the magnitude of the shunts. Two such triangles can be constructed from one abnormal curve (Fig. 25). If indicator traversed only one or other pathway, the

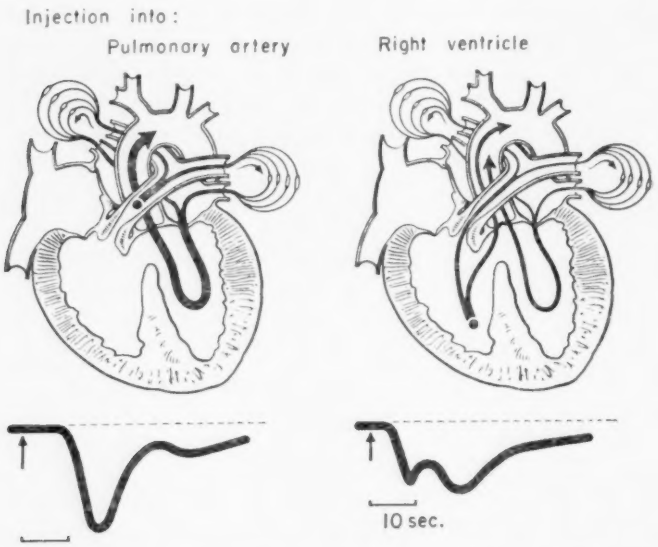

FIG. 25. Diagram illustrating method of localizing a right-to-left shunt by means of indicator-dilution curves. Increased indicator concentration denoted by a downward deflexion. There is pulmonary stenosis and a right-to-left shunt via a ventricular septal defect. After injection into the pulmonary artery all the indicator travels through the pulmonary circulation and a normal dilution curve results (left curve). When injected into right ventricle or further upstream the double-hump curve is due to passage of some indicator directly through the defect, while the remainder passes through the pulmonary circulation. (From Wood, Swan and Helmholz, 1957.)

forward triangle would represent the first part of a complete dilution curve. Study of each forward triangle thus provides information about the distribution of indicator along each pathway. The first triangle is constructed with one side extending from the appearance time to the time of the peak concentration value of the first hump on the upslope. The second side is the peak concentration value of his hump. One side

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of the second triangle is the peak concentration value of the second component of the abnormal curve. The appearance time of this latter component cannot be determined directly, but is estimated from the empirical relationship:

$$t_a/t_{C_p} = 0{\cdot}56 \pm \text{s.e. } 0{\cdot}007$$

where t_a = appearance time;
t_{C_p} = time of peak concentration after injection of indicator;

the time base of the triangle is then constructed from this point to the time of the second concentration peak.

In the quantitative estimation indicator should be injected close behind the region of the shunt. The lower part of the superior vena cava, or the right ventricle thus constitute satisfactory injection sites in the example in Fig. 25. The amount of indicator I injected, may be considered to be partitioned into a component i which passes to the sampling site via pathway (2), while the remainder passes via pathway (1). The proportion of shunt flow to total flow thus depends on the estimation of the ratio i/I. This is given by the relative areas of the two forward triangles as follows

$$i/(I - i) = k_1 \Delta_1/k_2 \Delta_{11}$$

where k_1 and k_2 are the proportionality constants relating the areas of the forward triangles; Δ_1, Δ_{11}, to the area of the whole curve (p. 143);

$$\text{therefore shunt flow/total flow} = k_1 \Delta_1/(k_1 \Delta_1 + k_2 \Delta_{11}) \quad . \quad . \quad . \quad (14)$$

In their formulation, Swan *et al.* make the implicit assumption that $k_1 = k_2$, and calculate the proportion of shunt as $\Delta_1/(\Delta_1 + \Delta_{11})$. The results calculated in this way were compared with estimates of the proportion of the shunt, obtained by blood-gas analysis (Fig. 26). The values obtained by the indicator method systematically underestimated the magnitude of the shunt. This can be explained by considering the difference in distances between injection and sampling sites resulting from the different traversal pathways of the two portions of injected indicator. It was seen on p. 139 that the asymmetry of the curve diminishes as the distance between injection and sampling site increases, when injecting into the central circulation. Thus, the proportionality constant relating the area of the forward triangle to the area of the whole curve cannot be the same, when the path lengths of the two portions of the indicator curve are clearly different. In the more asymmetrical distribution resulting from traversal of pathway (2), the forward triangle must represent a smaller fraction of the area of the

whole curve. Since the two path lengths probably differ by a factor of 2, a difference of about 20 per cent in the value of the proportionality constant could be expected (Fig. 11) and this would account for the discrepancy.

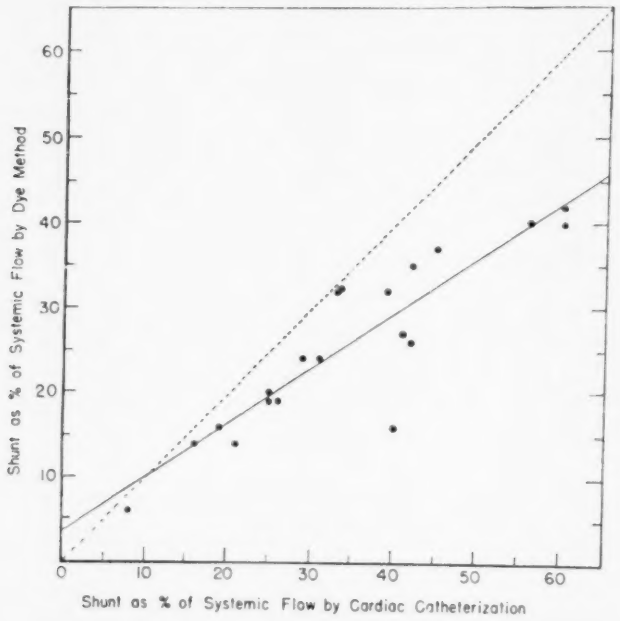

FIG. 26. Comparison of estimates of right-to-left shunt by the dye dilution method and by blood-gas analysis. The dotted line is the line of identity, and the firm line is the calculated regression line. (From Swan *et al.*, 1953.)

3. *Central arteriovenous (left-to-right) shunts*

3.1. *Qualitative changes in the curves.* The pathological basis underlying this type of shunt includes ventricular septal defects, atrial septal defects, patent ductus arteriosus, anomalous pulmonary venous drainage, etc.; the functional result is similar in all cases and these patients have a pulmonary blood flow that is higher than the blood flow through the systemic circulation.

The changes in dispersion of indicator are illustrated in Fig. 27. In the normal circulation all indicator leaves the left ventricle rapidly via the aorta, but in the presence of a shunt only part of the indicator is transported in the first instance along this pathway. The remainder passes through the ventricular septal defect into the right ventricle, traverses the pulmonary circulation, and enters the left ventricle once

again. The upslope of the curve is quite sharp and well defined, but the downslope is irregular and cannot be extrapolated by the Hamilton method.

The observations made by Swan *et al.* (1956a) in patients with atrial septal defects are of great interest from the point of view of non-uniformity of blood-flow distribution. When dye was injected into the

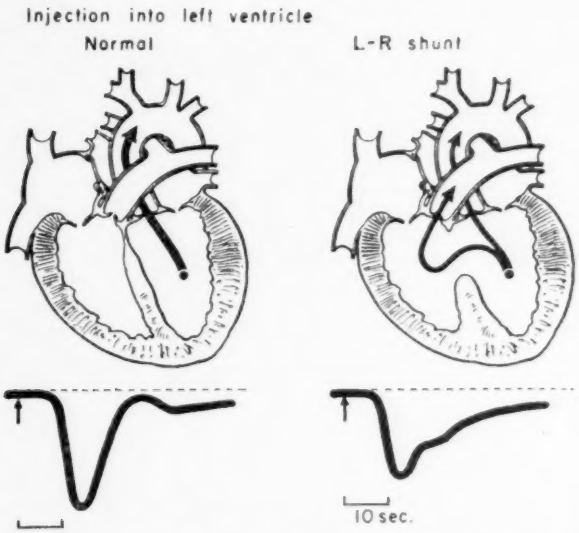

FIG. 27. Demonstration of the presence or absence of a left-to-right shunt at or distal to the left ventricle. The dilution curve below each schematic diagram is that which would be recorded from a distal peripheral artery. The vertical arrow on the dilution curve indicates the time of injection and increasing indicator concentration is represented by a downward deflexion. In the left-hand panel the ventricular septum is intact, and the indicator dilution curve is normal. In the right panel there is ventricular septal defect with left-to-right shunt, and the indicator curve shows the characteristic lowering of the peak concentration value, and the irregularity in the downslope. (From Swan and Wood, 1957.)

left pulmonary artery, the curves obtained were of relatively normal contour apart from the irregularities on the downslope (Fig. 28). After injection into the right pulmonary artery, the dilution curve had two concentration peaks. The first of these was usually small in magnitude, and coincided in time with the main deflexion that resulted from injection of dye into the left pulmonary artery. The second deflexion was usually larger, and followed the first after an interval of 4–6 sec.

The findings were interpreted as indicating a moderate degree of shunting of the blood flow of the left lung, but a very marked degree of shunting of the blood flow of the right lung. These findings were observed in the great majority of patients with atrial septal defect. Necropsy studies demonstrated the juxtaposition of the defect with the entry of the right pulmonary veins into the left atrium and this seems to provide a satisfactory explanation of the results.

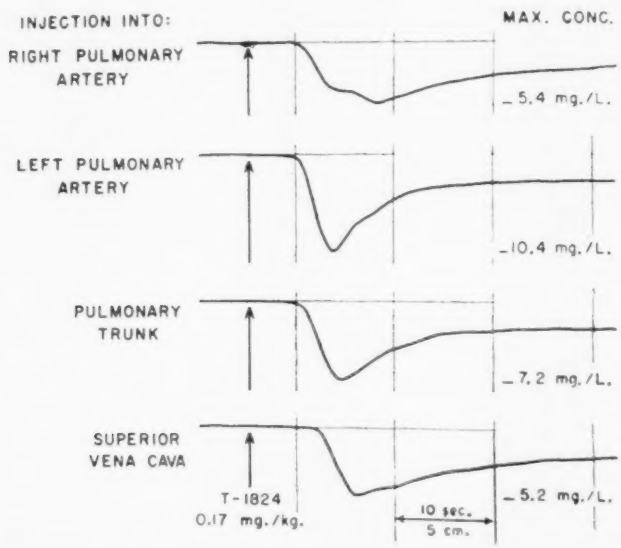

FIG. 28. Systemic indicator dilution curves recorded by means of an earpiece oximeter in a patient with atrial septal defect. Increasing dye concentration is represented by downward deflexion of the instrument. Injection was carried out in the right pulmonary artery, left pulmonary artery, pulmonary trunk and the superior vena cava as indicated on the left-hand side of each record. The maximum concentration values recorded on the right-hand side of each curve were obtained from curves recorded simultaneously at the radial artery, and serve to indicate the relative sensitivity of the instrument. (From Swan, 1956b.)

Silver *et al.* (1956) confirmed the above observations in dogs with experimental atrial septal defects. In these animals the direction of the shunt was predominantly from the left atrium to the right atrium, but in addition there was a small amount of shunt in the reverse direction. They showed that the right-to-left shunt was due almost entirely to the blood flow returning to the right atrium via the inferior vena cava; in most cases the blood flow returning from the superior vena cava did

not contribute to this shunt. This demonstration of preferential pathways of flow is very convincing proof that uniform mixing of blood does not occur in either atrium. While this might be considered to apply only to these pathological circulatory disorders, the well-known observations about the variability in oxygen content sampled from different parts of the normal right atrium, indicate that mixing is not uniform in the normal circulation.

3.2. *Quantitative estimation.* Fig. 28 demonstrates that the dispersion of indicator is increased in left-to-right shunts. In the curve obtained after superior vena caval injection, the irregularities of the downslope are not unduly prominent. This might suggest that the systemic blood flow could be estimated from the area of the curve, whilst the pulmonary blood flow could be estimated from the variance of the curve. This analogy with the quantitative estimation of valvular incompetence was suggested by Korner and Shillingford (1956), but subsequent detailed analysis of the limiting conditions of the dispersion method by Korner *et al.* (1959) makes it certain that the dispersion method cannot be applied to this type of shunt in the same way as in valvular incompetence. In valvular incompetence acceleration and retardation of indicator particles is a phasic process with each heart beat; on the other hand in central left-to-right shunts the discontinuity of division of indicator particles results in irregularities of the downslope of the curve. It is thus only the first part of the curve that permits estimation of the pulmonary blood flow. The method developed by Wood and his colleagues, which is very similar to the multiple-sampling method discussed in relation to valve incompetence, can be much more readily applied to this problem, and the difficulties due to proximity of injection and sampling sites are largely avoided.

Figure 29 illustrates the principle of the method in a patient with ventricular septal defect (Wood *et al.*, 1958). Indicator is injected into a lobar pulmonary artery and sampled (1) at a systemic arterial sampling site, and (2) at the right ventricular outflow tract just below the pulmonary valve (or alternatively the main pulmonary artery). All the injected indicator, I, travels to the left ventricle and is diluted by the pulmonary blood flow. The pulmonary blood flow can thus be estimated from the forward triangle of the systemic arterial curve according to equation (9). The fraction i of indicator that passes through the defect is represented by the forward triangle of the curve recorded from the right ventricular outflow tract. The ratio i/I thus represents the proportion of pulmonary blood flow that traverses the shunt and is estimated from the respective forward triangles

$$\text{shunt flow/pulmonary flow} = k_1 \Delta_1 / k_2 \Delta_{11} \quad . \quad . \quad . \quad (15)$$

where Δ_1 forward triangle of the right ventricular outflow tract curve;

Δ_{11} forward triangle of the systemic artery curve.

Wood *et al.* (1958) and David *et al.* (1958) obtained good estimates of the magnitude of the shunt with the implicit assumption that $k_1 = k_2$ (Fig. 30). This assumption seems reasonable, since the distance between the injection site and the two sampling sites does not differ greatly.

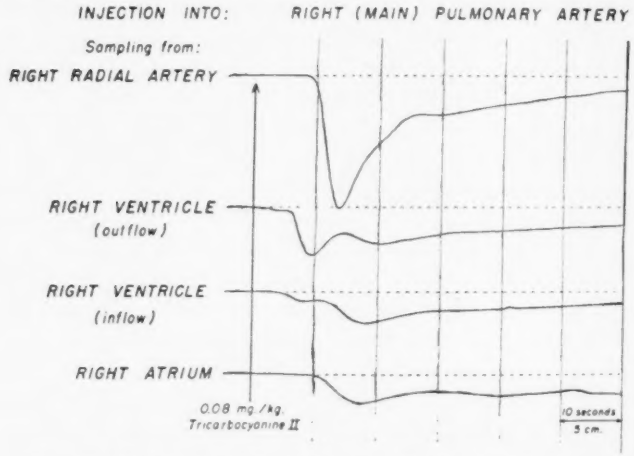

FIG. 29. Demonstration of the use of indicator dilution curves recorded from a systemic artery and selected sites on the right side of the heart, to localize a left-to-right shunt to the outflow tract of the right ventricle. Dye appeared in the right ventricular outflow tract before it appeared at the systemic artery denoting the presence of a left-to-right shunt. Since no early dye appeared in the right atrium or right ventricular inflow tract the shunt is localized to the outflow tract of the right ventricle. The calculation of the magnitude of the shunt is described in the text. (From Wood *et al.*, 1958.)

The results of David *et al.* showed good agreement between the absolute flow values calculated from equation (9), and the direct Fick method. The scatter in estimates of pulmonary blood flow was less than that obtained by Ramirez de Arellano *et al.* (1956), where there may have been greater variation between individuals leading to greater differences in the value of the proportionality factor k in equation (9). In general the indicator dilution curves are more important in the estimation of the *proportion* of the shunt than in calculating absolute flow values, since the pulmonary blood flow can always be determined in these patients by the direct Fick method. Under these conditions the problem of accurate dimensional specification of the system assumes less importance than in valve incompetence.

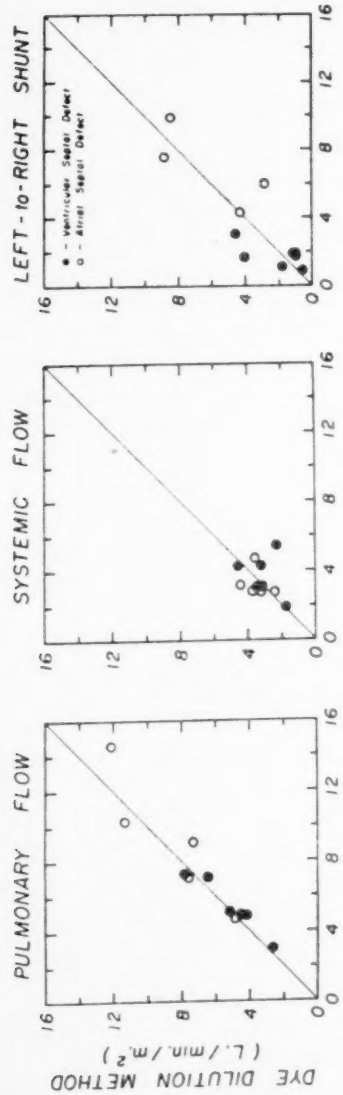

FIG. 30. Comparison of estimates of flow obtained by the dye dilution (forward triangle) method and by the direct Fick method in eleven patients with ventricular septal defect(●), or atrial septal defects (○). (From David *et al.*, 1958.)

It should be noted that the chief difference between a central left-to-right shunt and valvular incompetence is that in the former case physical separation of indicator particles occurs, but in the latter case they are merely displaced in time. The dispersion method and the multiple-sampling method are thus complementary applications of the same underlying principle.

VIII. Conclusion

The chief reason for the original gain in popularity of the indicator-dilution method over the direct Fick procedure was that it permitted measurement of cardiac output without the necessity for cardiac catheterization. Unfortunately, this becomes essential when the indicator method is applied to the study of dispersion, since the flow conditions in peripheral veins obscure the relationship of the indicator distribution to events occurring in the central circulation.

The results discussed in this article demonstrate that mixing in the circulatory system cannot be regarded as a uniform process. The characterization of the indicator dilution curve by two near-Gaussian components has provided a qualitative picture of the dispersion process which is in good agreement with the experimental facts. This concept explains the change in the symmetry of the curves with changing distance between injection and sampling sites. When a very small distance between the two sites is used, this has the advantage of minimizing the likelihood of any hidden recirculation. However, moment-to-moment variations in the "axial" and "boundary" components of the distribution result in far greater irregularities in the curves, than if the distance between the two sites is larger. In effect this sets a limit on the quantitative application of the method to the direct study of dispersion in small anatomical components of the circulation. It seems that the concept of optimum distance between injection and sampling sites has received insufficient emphasis in the past.

There seems little doubt that it will be possible to extend studies of circulatory mixing in specific parts of the circulation by the use of the indicator method. The development of the methods used by Gonzalez-Fernandez *et al.* on the effects of the sampling system on the curve, obviously have general applications to the above problems without requiring very close distances between injection and sampling sites. By using multiple injection and sampling sites it should be possible to "recover" the curve due to any component of the circulatory system, and these developments foreshadow the use of more elaborate methods of analysis than have been employed up to date.

At present the use of relatively simple methods has provided means

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of diagnosis and quantitative estimation of a variety of circulatory disorders. It seems that enough is now known about the advantages and limiting conditions of the various methods so that their application to physiological problems in the intact circulation should soon provide more information about the haemodynamics of these disorders.

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THE BUOYANCY OF FISH AND CEPHALOPODS

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THE BUOYANCY OF FISH AND CEPHALOPODS

E. J. Denton

I. Introduction

Neutral buoyancy, that is having the same density as sea water, will evidently be a great advantage to a pelagic animal. An active control of buoyancy will be even more useful. Man-made machines for travelling in mid-water, such as the submarine and bathyscaphe, are arranged not only to stay at a chosen depth with little effort, but also to use changes in buoyancy to move from one depth to another. In the submarine this is done by filling some chambers with either air or water (a method used also by the cuttlefish). In the bathyscaphe the weight in sea water of the observer's steel sphere is approximately balanced by a large flotation chamber containing a liquid less dense than sea water (a method used by the cranchid squid). The bathyscaphe is made a little denser than sea water to allow it to descend in the sea and the engineer simply drops off metal ballast when he wishes to come up again.

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It is difficult to calculate how much saving of effort neutral buoyancy can give. An active pelagic fish deprived of the gas in its swimbladder would have a reduced weight in water of 5–8 per cent of its weight in air (depending on whether it were a marine or a freshwater fish) and would have to exert a corresponding force downward on the water if it wished to stay at one level. A few per cent of the body weight may seem quite a small load for an animal to bear, but we must remember that a weight easily supported on land can only be sustained in water by continuous movement. We can imagine that a 60 kg man would find it very tiring to hold up 3 kg of lead by treading water all his life but an animal like the common mackerel has a similar buoyancy problem. Although much better designed for swimming than man even an active pelagic fish *very infrequently* exerts a force of over 25–50 per cent of its weight (Gray, 1953) so that to exert *continuously* a force of a few per cent of its weight will be a formidable task (see Denton and Shaw, quoted by Denton and Marshall, 1958). In aquaria, animals such as the common prawn, plaice and dogfish, which are known to be only a little denser than sea water, can be seen to work hard whenever they wish to stay off the bottom. Far from being

surprised then by the ingenuity in design which allows many animals to achieve neutral buoyancy, we ought to be astonished to find, as we do indeed find, that many successful animals are far from neutrally buoyant and swim all their lives to stay in the mid-waters.

One fruitful way of thinking of the buoyancy of an animal is to draw up what might be described as a buoyancy balance sheet, putting on one side those components which are denser than sea water and which will therefore tend to "sink" the animal and on the other side the components which are less dense than sea water and so will tend to "float" it.

The principal "sinking" components of animals are their proteins and skeletons. The effective densities in solution given for a number of proteins by Höber (1945) are all close to 1·33. Such a density would account for the fact that the muscles of common edible fish and squid, which contain about 20 per cent by weight of protein, are about 5 per cent denser than sea water. Skeletons are often made of calcium salts of density close to 3, and sometimes of chitin which is also denser than sea water; the chitinous pen from *Loligo* has a density of 1·2. The relative proportions of protein and skeleton vary with the animal. In a muscular animal like the squid *Loligo*, or a common coastal fish the protein of the muscles will be the most important "sinking" component; in *Nautilus* and the pelagic *Foraminifera* the minerals of the shell will have a much greater weight in sea water than the living tissues of the animal.

The principal "floating" components of animals are fat, body fluids which are less dense than sea water, and chambers filled with gases.

Fat whose density is generally close to 0·9 will give some buoyancy and in some fish and in some cetaceans (Krogh, 1934) the fat content is high and the upthrust which it gives is very appreciable. Fat is certainly the principal variable in deciding the buoyancy of a man (Behnke, 1942).

In fresh-water animals the tissue fluids are more salty and denser than the surrounding water but in marine animals the tissue fluids are either isotonic or hypotonic to sea water and often they can give some net lift. Their contribution to buoyancy, although of minor importance in very muscular animals, could be vital in very watery beasts like the ctenophores and the medusae. These contain very little protein and the possession of body fluids which are slightly less dense than sea water may be their sole buoyancy device (Marshall, 1954). Oceanic waters have a density of around 1·026 and if an animal's body fluids were replaced by pure water this would give a net lift in the sea of over 2 per cent of the animal's weight in air. For an animal to have tissues without salts is clearly impossible, but fish often

maintain tissue fluids with less than 50 per cent the osmotic strength of sea water (Krogh, 1939) and they will gain some buoyancy from these fluids.

Even with tissue fluids isotonic with sea water an animal can gain some buoyancy by changing the *kind* of ions and molecules which its body fluids contain. Gross and Zeuthen (1948) in their work on the diatom *Ditylum* give a table (Table 1) showing the effect on density of changing ions without change in total osmotic concentration. They studied solutions which were isotonic with sea water of density 1·0235.

The proportions of salts were those present in sea water (Harvey, 1945). All the solutions were isotonic with one another. Solution V represents simplified artificial sea water.

TABLE 1

Specific Gravity of Salt Solutions Isotonic with Sea Water of Salinity 32·5 0/00 (*after* Gross and Zeuthen).

Solution	*Salts present*	*Specific gravity at 22°C*
I	NaCl	1·0199
II	NaCl, KCl	1·0201
III	NaCl, KCl, $CaCl_2$	1·0211
IV	NaCl, KCl, $CaCl_2$, $MgCl_2$	1·0211
V	NaCl, KCl, $CaCl_2$, $MgCl_2$, Na_2SO_4	1·0235

From this table we see that some gain in buoyancy can be made by changes in the relative concentrations of the common ions of sea water and that it is particularly helpful to exclude the divalent ions, calcium and sulphate.

A much greater buoyancy gain can be achieved by replacing the common ions of sea water by other substances. A high concentration of hydrogen ions in an animal would help greatly but such a high concentration has not yet been found, nor indeed does it appear likely. High concentrations of ammonium ions, which appear almost equally improbable, are however used. A solution isotonic with sea water of density 1·026 but containing four-fifths ammonium chloride and one-fifth sodium chloride has a density of about 1·010. The clearest quantitative example of the use of ammonium ions for buoyancy is that recently found in the cranchid squid (Denton *et al.*, 1958) but Goethard and Heinsius (1892) (see also Krogh, 1939) gave cogent reasons for believing that the protozoan *Noctiluca*, which is often found floating at the surface of the sea, uses a high concentration of ammonium

ions to give it buoyancy. Harvey (1917) and Gross and Zeuthen (1948) found this explanation of the high buoyancy of *Noctiluca* an improbable one, and Iida and Iwata (1943) were not able to confirm that *Noctiluca* cell sap did contain high concentrations of ammonium. It is very difficult, however, to put any simple interpretation on Goethard and Heinsius's results other than that which they themselves gave; perhaps Iida and Iwata caught animals in a different condition.

In other animals there may be other substances which play a role similar to that which ammonium plays in the cranchid squid. The high concentration of urea found in the tissues of elasmobranchs seems a possibility, but urea solutions give only a very slight positive buoyancy over sea water. A solution of trimethylamine hydrochloride is appreciably less dense than an isotonic solution of sea water, and might perhaps be used to give some lift (E. J. Denton and T. I. Shaw, unpublished measurements).

At 1 atm pressure air has a density of only about $\frac{1}{800}$th that of water, and gas spaces offer the most obviously effective way of giving an animal or a plant upthrust. The bladders of seaweeds and fishes and the chambered shells of the cuttlefish and *Nautilus* are amongst the number of such gas spaces and they offer some of the best puzzles to be found in physiology. The beautiful blue "sail" of the Portuguese Man-of-war, *Physalia*, is also a gas filled flotation chamber. It might be thought that the creation of such a chamber at the surface would not be difficult, but even here we find something very surprising; Wittenberg (1960a) has shown that this float contains not a gas mixture rather like air as was once thought (Schloesing and Richard, 1898), but a considerable proportion (up to 5 per cent) of carbon monoxide.

If a gas space is to be maintained at depth in the sea then three methods for its preservation seem to be possible.

(1) If the walls of the chamber containing gas are not rigid the gas pressure inside the chamber must match the external hydrostatic pressure. Some special tricks will then be needed both to secrete gases and to prevent these gases going into solution. This method is used by some fishes down to great depths and against enormous pressures.

(2) A gas space may be created by the active removal of liquid from the inside of a rigid-walled "box". The gases inside the box can then come to equilibrium with the gases dissolved in the tissue fluids surrounding the box. Since the gases dissolved in the sea are at all depths approximately in equilibrium with the atmosphere, they will probably exert a pressure of close to 1 atm. With this method the rigid wall of the box must be capable of bearing all the external pressure except for the 1 atm balanced by the gases which it contains. At 100 m

depth the box must stand 10 atm pressure; at 1000 m depth it must stand 100 atm pressure. A method such as this is used by the insect larvae *Corethra* (Krogh, 1911) in very shallow waters and against slight pressures, and it is also used by the cuttlefish *Sepia* at fair depth.

(3) Finally, an animal might fill a rigid-walled box with air at the surface, seal it completely so that water cannot enter, and then use it down to the depth at which the box would implode.

We may, of course, imagine combinations of these methods. An animal might pump gas into a rigid chamber and so expel the liquid which it contains, seal the chamber to water and then use it to give buoyancy at much greater depths. The pressure of gas inside the box would not then need to match exactly the external hydrostatic pressure, and the box could be much less strong. Suppose, for example, the gas pressure inside the box were 50 atm and the external hydrostatic pressure 75 atm, the box would have to stand only the difference in pressure of 25 atm. Some such system was suggested by Bruun (1943) as a possible mechanism for the shell of the cephalopod *Spirula*.

If an animal's buoyancy chamber is permeable to water and contains gas at only low pressure, then, if it is not to fill with water and become useless for buoyancy, there must be some pumping mechanism capable of holding out water against the maximum pressures to which the animal is subjected.

As there already are some instructive earlier accounts such as those in Murray and Hjort (1912) and Marshall (1954) and a major review on buoyancy by Jacobs (1935) we may, after this brief introduction, turn to a discussion of the buoyancy of fish and cephalopods. Here considerable advances in our knowledge have been made, even since the fine review on the swimbladder of fish by Harden Jones and Marshall (1953).

II. FISH

1. *Fish with a gas-filled swimbladder*

1.1. *General properties of the swimbladder.* To a physiologist the swimbladder, a very highly specialized organ, is a most promising field of study. It has already attracted a number of distinguished physiologists including Borelli, Johannes Muller, J. S. Haldane and Krogh, but still continues to offer problems of great interest. We shall discuss only the principal function of the swimbladder which is quite clearly hydrostatic, leaving aside those adaptations which allow it to be used in sound production, sound detection and as an oxygen reserve (Harden Jones and Marshall, 1953).

Bottom-dwelling fish like the plaice, which have no gas-filled swimbladder, are about 5 per cent denser than sea water and they can only

remain off the bottom by great and continuous effort. All the common marine fish which can hover and manoeuvre gracefully possess a gas-filled swimbladder of about 5 per cent of their total volume and are very close in density to sea-water. Now water, which only contracts about 1 part in 20,000 per atm and the organic components of animals are not very compressible and their densities will not be much changed with depth. Gases, on the other hand, obey Boyle's law fairly well and, unless constrained in some way, the gas in the swimbladder will change in volume rapidly with depth, for example doubling on going from 10 m to the surface of the sea. It is true that a perfectly immobile fish could be in equilibrium at one depth but, like a Cartesian diver, the slightest deviation up or down would send it further upwards or downwards with ever increasing velocity. The manifest inconvenience of such changes in buoyancy to a fish led the earlier workers to suppose that the muscles around the swimbladder must control its volume, holding it at the equilibrium value when the fish wished to stay at one level. Borelli believed the swimbladder to be also an organ of locomotion, the muscles being actively constricted when the fish wished to descend and relaxed when the fish wished to rise. Dispute later arose between the supporters of Borelli's views and those who, with Delaroche, thought that the muscles around the swimbladder were used only to hold its volume constant. These very rational differences of opinion as to just *how* the muscles control the volume of the swimbladder were interrupted by Moreau's elegant experiments (1876) showing that they exert no control whatsoever. Surprisingly enough, fish *are* in a state of unstable equilibrium, and compensate for transient changes of pressure by swimming. Moreau showed that when a fish *voluntarily* changes depth, even by a very small distance, the volume of gas within the swimbladder simply follows the change in hydrostatic pressure as if there were no other constraints on it. If it swims to a slightly higher level and stays there a short while, the gas expands progressively with the upward movement and then remains at its increased volume. This change does not, as Borelli's hypothesis would predict, precede the movement and neither is it abolished at the end of the movement. Even the gurnards which have particularly well-developed muscles in the swimbladder wall were shown to use these muscles, not for controlling its volume, but for making their well-known grunting sounds (Moreau, 1876).

One or two interesting qualifications can now be made to Moreau's hypothesis, but these in no way disturb its validity. Alexander (1959a, b, c, d) has recently shown that in the Cyprinidae the swimbladder does *passively* resist the expansion of the gas within it if the external pressure falls; but for twenty-two other species he found that the swimbladder obeyed Boyle's law remarkably well. The sea horse *Hippocampus*

brevirostris has recently been shown to use the muscles of its swimbladder wall to control not its density but its posture (Peters, 1951). The swimbladder is divided into anterior and posterior chambers. When the sea horse swims with its body horizontal the posterior chamber is expanded and the anterior chamber small; when the fish becomes upright, with its tail attached to some object, the anterior which is now the upper chamber becomes larger and the posterior chamber smaller. Such changes take about 10 min and are evidently brought about by the contraction and relaxation of the muscles surrounding the anterior chamber.

To Moreau fell also the distinction of demonstrating that, in response to longer-lasting changes in pressure, the fish can change the *mass* of gas within the swimbladder to keep its volume constant (the pressure of the gas always being equal to the external hydrostatic pressure). In one of his experiments fish which had lived at the surface of the sea for some time were placed in baskets at 7–8 m depth. After a few days when brought to the surface they were found to have secreted extra gas into the swimbladder and to be appreciably less dense than sea water. On accepting Moreau's views it became easy to see why cod caught at 100 m depth in the sea and brought to the surface had very distended swimbladders and could not swim down again, whilst cod caught in shallow waters had no such difficulties. The nervous control of the secretion of gases was also one of Moreau's discoveries and interesting experiments on this control have been made by Bohr (1894) and Fänge (1953).

1.2. *Limits of performance of the swimbladder.* Clearly the depth limits with which it works and the maximum rates at which gas can be added to, or lost from, the swimbladder, will be important criteria on which we can judge theories of its mechanism.

Many fish possessing gas-filled swimbladders live in very shallow water and amongst their number we may list such common fish as the trout, salmon, perch and pike. One depth limit is then the surface, and here the pressure on the gas within the swimbladder will be a little over one atm. The maximum depth to which a fish can usefully employ a gas-filled swimbladder is less certain but we do know that this depth is very great. Thus *Nematonusus armatus* has been caught a number of times (particularly by the *Michael Sars* and the *Albatross*) between 4000–4700 m. In the report of the Swedish Deep Sea Expedition (Nybelin, 1957) there is a photograph taken on the deck of a ship, of a specimen caught between 4500 and 4600 m. It can be seen to have a very dilated swimbladder. At 4500 m the swimbladder of this fish must have contained *some* gas under a pressure of 450 atm. This does not prove that the volume of this gas was sufficient to make the fish

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neutrally buoyant at 4500 m for, although the gas left in the swimbladder was sufficient to make it very large at the surface, its buoyancy would have been negligible when reduced in volume about 450 times and its density increased to about 0·6. We may reasonably suppose however, that this swimbladder had burst on the long haul to the surface and lost most of the gas which it had contained before capture.

Strong evidence that the swimbladder can be functional at great depth comes from the observations of fish made from bathyscaphes. Thus Pérès *et al.* (1958) write of *Halosaurus johnsonianus* Vaillart seen at 2200 m. "Les specimens observée ici mesurent 50–80 cm de long et le plus souvent, nagent lentement contre le courant le corps disposé obliquement, la tête êtant a proximité du fond, parfois ils se disposent verticalement le museau a quelques centimètres de la vase et se laissent entrainer par le courant, en maintenant leur equilbre par de légers battements de queue". To swim in this way the fish must clearly be very nearly neutrally buoyant.

Observations such as these make it certain that swimbladders can contain gas, and *almost* certain that they can secrete gas, at depths where there is a hydrostatic pressure of several hundred atmospheres. From Marshall's (1960) wide survey we know that the distribution of fishes possessing swimbladders in the ocean is a very surprising one. About a half of the pelagic fish swimming in the upper reaches of the ocean (200–1000 m) have a highly developed gas-filled swimbladder whilst, from 1000 m down to almost the bottom, the swimbladder is either absent or regressed. At, or very close to the bottom, and this was very surprising until their habits were known, a great many of the fish have very well-developed and functional swimbladders.

Although the bottom-dwelling fish will have gas under the highest pressures, the greatest rate of change of gas content is to be sought in the pelagic fish in the top 1000 m. These fish frequently make extensive daily vertical migrations and, if they are to retain neutral buoyancy everywhere, they must secrete gas as they descend and reabsorb gas from the swimbladder as they rise. As we should expect from the obvious difficulty of studying these fragile oceanic fish, our information is very incomplete. Kanwisher and Ebeling (1957), making plausible assumptions, have calculated the minimum work which would have to be done to pump gas into the swimbladder to maintain constant volume during a migration to greater depth. The limits between which *individual* fish move is not known. The daily vertical migrations of fish were first recognized by studying the catches made with mid-water trawls at different times of day and night. It was found that fish which were caught only at depth in the day-time became common at the surface and less common in the deeper waters at night-time. Very

strong mid-water sound-scattering layers (the so-called deep-scattering layers) have recently been found by echo sounding, these layers move up at night and down in the day. As an example of such a layer we may instance one reported by Kampa and Boden (1957) which moved between 30 and 300 m. The sound scattering is thought to come largely from the swimbladders of fishes and the change in depth of these deep-scattering layers is thought to indicate the migration of fishes (Marshall, 1951). Kanwisher and Ebeling calculate that a very large amount of work in pumping gas would be required to maintain constant specific gravity for vertical migrations such as those indicated in these ways. They conclude that the regulation of buoyancy is unlikely to be perfect and that the specific gravity of these fish probably differs appreciably from sea water at either the top or bottom of their migration.

There remain, however, two indications that secretion and reabsorption of gases can take place at high rates in these fish. Some Myctophids (lantern fish) which almost certainly migrate downwards during the day, can be caught in hand nets from a ship's side at night. Fish so caught were found by Kanwisher and Ebeling to be in buoyancy equilibrium having a swimbladder volume at the surface of about 4 per cent of the fish's total volume. The composition of the swimbladder gas, with often about 90 per cent oxygen, was, however, typical of fish living at over 100 m depth (see p. 192). This suggests that gas is secreted at depth and largely reabsorbed when the fishes rise to the surface. Moreover, as we shall see later, the swimbladders of bathypelagic fish are very much more highly developed both for the secretion and reabsorption of gases than those of surface fish (Marshall, 1960). We may therefore be sure that the swimbladders of the bathypelagic fish have very high capabilities of gas secretion and reabsorption but we still cannot assign any limits to their performance.

1.3. *Structure of the swimbladder.* By a too exclusive attention to structure the earlier workers on the swimbladder were led astray, and indeed it seems a good general rule not to deduce function from a knowledge only of anatomy. But whilst important functions can sometimes be performed by undistinguished structures the swimbladder displays in parallel with its extraordinary capacities, a number of remarkable anatomical features. We may then search in the anatomy for those structures which allow it to retain gas when the fish is at a constant depth, to lose gas when the fish ascends and to secrete gas when the fish goes deeper.

We shall begin by considering the swimbladder of the common freshwater eel *Anguilla anguilla* which was particularly well studied by Woodland (1911) and by Krogh (1922). The eel's swimbladder is

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shown diagrammatically in Fig. 1(a). The wall is silvery and thought to be very impermeable to gases. Isinglass which is made from the dried swimbladders of some fishes, although used in Britain principally for the fining of beer, may also be used in cooking as a thin skin over food or coffee to retain aromas. In the eel the main gas-filled part of the swimbladder is connected by a tube, the pneumatic duct, to a valved opening into the oesophagus and through this opening gases can be lost if the swimbladder gases expand unduly.* The blood supply to the

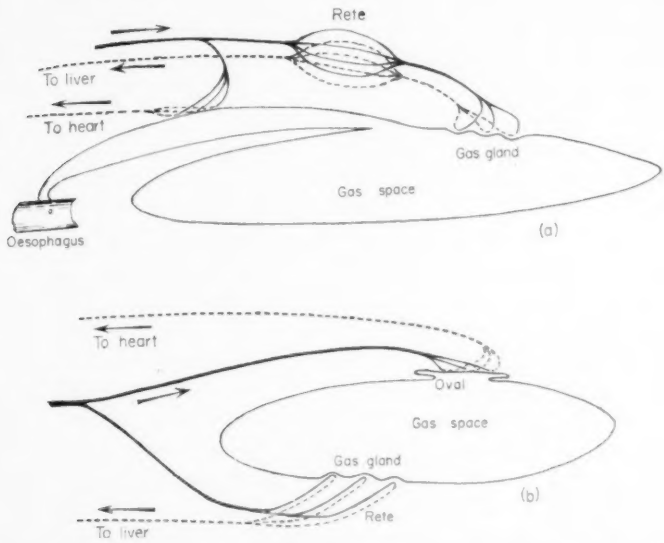

FIG. 1 (a) Diagram of the swimbladder of the eel *Anguilla anguilla*.
(b) Diagram of the swimbladder of the perch *Perca fluviatilis*.

swimbladder passes along the wall of the pneumatic duct, through the rete mirabile, which consists of two very conspicuous red bodies, to the secreting cells in the wall of the swimbladder which form the gas gland. The gas gland is clearly the gas-secreting organ for, in fish which are actively secreting gas into the swimbladder, the gas gland is often covered with a foamy liquid film. Bubbles have not been seen within the cells of gas gland and it has been suggested that they are only formed extracellularly perhaps in the clefts or ducts which lead into the lumen of the swimbladder (Fänge, 1953). However, it must be

* In other fishes the pneumatic duct is principally useful not in allowing gas to escape but as a channel by which air gulped into the mouth at the surface can be taken into the swimbladder.

remembered that the direct observations made on living secreting gas glands have not been of a kind which would allow the detection of small intracellular bubbles if such bubbles did exist. In the rete the arterioles passing to the gland break up into capillaries which come into intimate contact with corresponding venous capillaries arising from the venules coming from the gas gland. The capillaries of the rete are the longest known in nature; in the eel they are no less than 4 mm long, a very great length when we consider that those of muscles, otherwise amongst the longest, are seldom more than $\frac{1}{2}$ mm long. The rete consists of little but the two sets of capillaries (Fig. 2) and in an eel Krogh estimated that it contained 88,000 venous and 116,000 arterial capillaries. The blood going to the gas gland passes through no less than five sets of capillaries on its way round the body, those of the gills, arterial rete, gas gland, venous rete and liver.

The rete is probably of great importance both in the retention and secretion of gas. Haldane (1922) suggested that a high concentration of carbon dioxide was built up in the gas gland and that this carbon dioxide displaced oxygen from oxyhaemoglobin. He wrote "This arrangement (the rete) reminds us of that in a regenerating furnace where the heat carried away in the waste gases is utilized to heat the incoming air. . . . It seems probable therefore that the function of the rete mirabile is to enable venous blood to communicate part of its carbon dioxide to the arterial blood. The effect of this will be to raise the carbon dioxide pressure of the blood supplied to the gland and so raise the oxygen pressure. There may be active secretion of carbon dioxide into the arterial capillaries; and this hypothesis is supported by the existence in the arterial capillaries of a very peculiar thickened endothelium figured clearly by Woodland." This particular mechanism of gas secretion dependent on carbon dioxide is no longer generally accepted, but Haldane's general ideas remain the basis of the modern views of rete function. It is still thought that secretion from venous to arterial capillaries may play an important role in the building up of high concentrations of some substances at the gas gland; it is still universally agreed that the counter-current diffusion system of the rete will prevent the rapid loss of oxygen and nitrogen from the swim-bladder, where these gases exist at high partial pressures, into the general circulation where their combined partial pressures will be equal to about 1 atm.

A second and more common type of swimbladder is shown in Fig. 1(b). The retial capillaries forming a counter-current diffusion system still exist but they are now continuous with the capillaries of the gas gland. There is no reason to suspect that there is any functional difference between such a "unipolar" rete and the "bipolar" rete found in the

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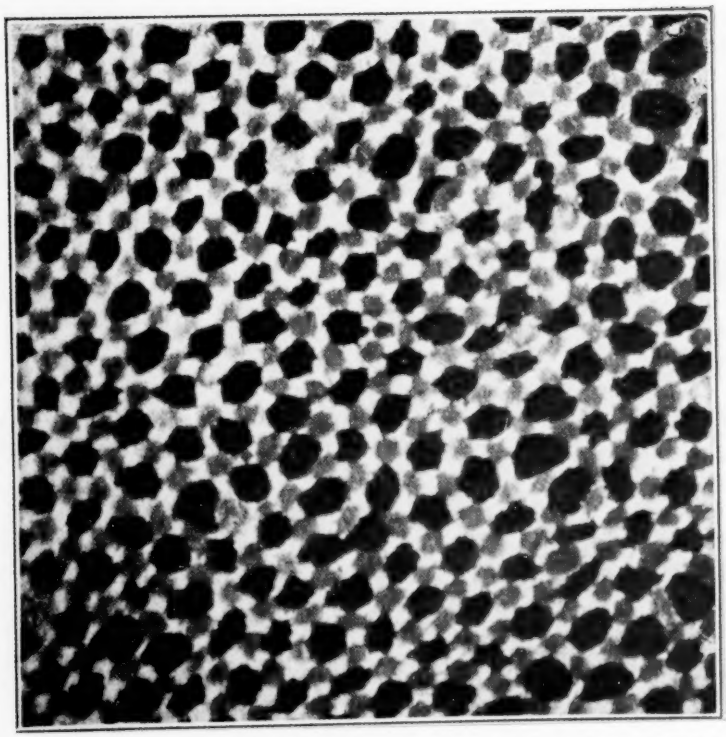

FIG. 2. *Anguilla anguilla*. Transverse section of rete mirabile. Arterial capillaries grey, venous capillaries black. (After Krogh, 1922.)

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eel. A more important difference is the absence of a pneumatic duct or, indeed, of any opening to the exterior. This difference is the basis of a general classification of fishes into the physotomes which have a pneumatic duct, and the physoclists which have none. It will be remembered that the pneumatic duct enabled the physotomes to lose excess swimbladder gas. In the physoclist this role is sometimes taken over by a structure discovered by Jaeger (1903) which is called the "oval". Here, one region of the swimbladder wall (Figs. 3 and 4) can either be exposed to, or occluded from, the gases of the swimbladder by the action of a

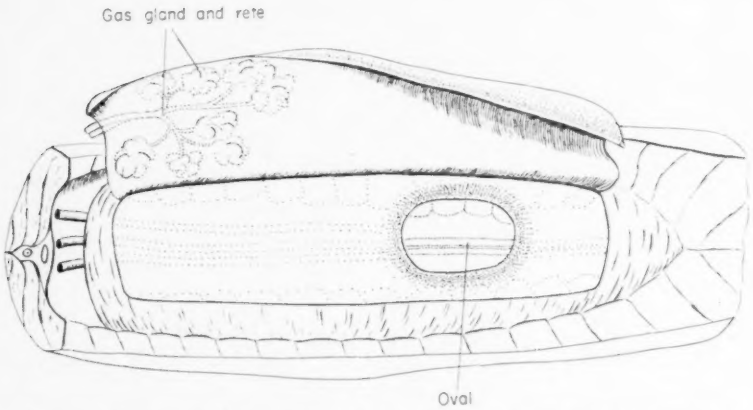

FIG. 3. Drawing of the swimbladder of the perch. The swimbladder wall has been cut and its ventral surface reflected back to show the gas gland and the oval. (After Jacobs, 1935.)

ring of muscle around its perimeter. This region has a rich blood supply which, unlike that to the gas gland, does not come through the rete. When the swimbladders of such fish are unduly expanded they have open ovals and lose gas. This is what might be expected for, if the oval is open, a gas present in the swimbladder at a higher partial pressure than in the blood will dissolve in the blood stream and be carried away in the oval's circulation. Fish which are in buoyancy equilibrium do not lose gas and they are found to have closed ovals. In such a physoclist the retention or loss of gas can then be decided by the closing or opening of the oval. Other physoclists have no oval but have instead a part of the swimbladder which can be cut off from the rest by a transverse diaphragm and Fänge (1953) and Marshall (1960) give interesting accounts of the differences of swimbladder structure in different fishes.

An analagous system to the oval may even be used in physotomes for the wall of the pneumatic duct, like that of the oval, has a blood supply

which does not pass through the rete and which may therefore be used for the reabsorption of gases. In the conger eel E. J. Denton and T. I. Shaw (unpublished observations) have noted that when the fish is in buoyancy equilibrium, the pneumatic duct is flattened between the wall of the swimbladder and an outer coat which surrounds both

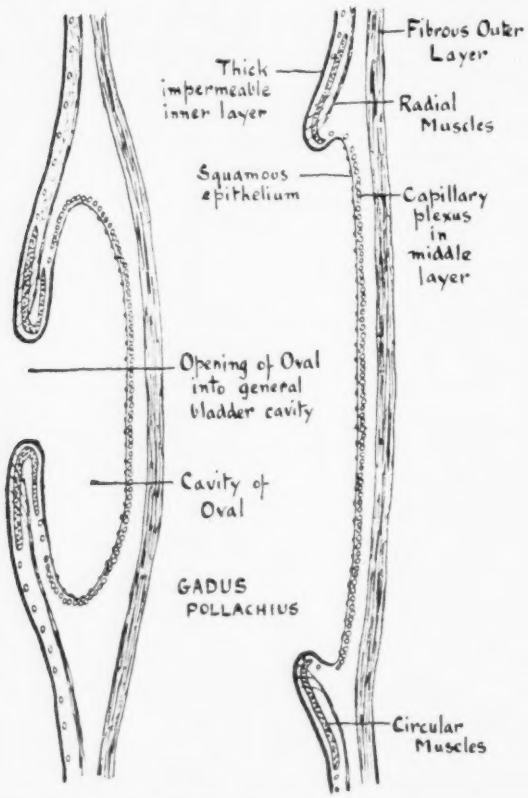

FIG. 4. Drawing of the oval of the pollack. Left side, semi-closed oval. Right side, open oval. Diagrammatic figures constructed from sections. (After Woodland, 1910–13.)

pneumatic duct and swimbladder. The duct is effectively valved at both ends. When the external pressure is a little reduced or the internal pressure a little increased (thus increasing the volume of the swimbladder) the extensibility of the various coats of swimbladder and pneumatic duct is such that the pneumatic duct is suddenly opened and quickly fills with gas. It is only at a much greater volume of swimbladder (about twice the value which gives the conger neutral

buoyancy) that the external valve opens and gas is lost through the mouth by way of the oesophageal opening. This mechanism would allow the pneumatic duct to function as an oval for small increases in volume of the swimbladder but as a true safety valve for large increases in volume.

There is no doubt at all that physostomes survive very rapid changes of depth very much better than physoclists. Most common physoclists, such as cod, whiting and hake are killed by the damage done by an expanding swimbladder when they are caught and hauled to the surface in a trawl; but conger eels survive such a change of depth perfectly well. The great majority of marine fish including deep-sea species are nevertheless physoclists and have evidently not been designed with trawling in mind. Harden Jones and Marshall see the pneumatic duct as being of especial use for shallow-living fish who can fill their swimbladders with air gulped in through the mouth at the surface.

Although the great majority of fish have the counter-current system of the rete mirabile through which blood must pass on its way to the gas gland, some physostomes, notably the Coregonidae which live at the bottom of deep lakes, have generally been thought to have no rete, and for this reason these fish have received a good deal of attention. Fahlen (1959) has recently shown however that *Coregonus laveratus* has a diffuse rete mirabile. Since this species has been regarded as typical of the Coregonidae it is clear that the histology of the swimbladder of other species of Coregonus, and other salmonids needs re-examination.

1.4. *The secretion of gases by the swimbladder.** To a physiologist the main interest of the swimbladder is the *secretion* of gases. The reabsorption of gases through the oval or their loss through the pneumatic duct demands only properties which are common to many other tissues and many animals.

The extent of the problem of secretion was announced, literally, with a bang. In 1803 the distinguished French scientist M. Biot noted that fish caught by line at great depth came to the surface with their swimbladders sometimes projecting from the mouth and their bodies distended with gas. He determined to analyse the gas and introduced some of it into a glass eudiometer tube together with an excess of hydrogen. On passing a spark there was a violent explosion which broke his instrument, and he realized at once that the sample of swimbladder gas must have contained a very much higher concentration of oxygen than is present in air. With a new instrument he was able to show that, whereas the gas from the swimbladders of fish living

*In accord with a common usage a gas is described as being secreted when it is accumulated in the swimbladder to a higher partial pressure than in the blood.

near the surface often contained less oxygen than air, the proportion of oxygen increased with depth and that gas taken from fish living at appreciable depths contained almost pure oxygen. It thus became clear that some swimbladders must be capable of secreting oxygen against very high pressure gradients (Biot, 1807).

Almost a century after Biot's experiments had demonstrated that the swimbladder could concentrate oxygen to many atmospheres pressure Hüfner (1892) found that the swimbladders of whitefish (*Coregonus*) caught at 60–80 m in Lake Constance contained almost pure nitrogen. On being brought to the surface these fish were bloated with enough gas to have made them approximately neutrally buoyant at the bottom of the lake so that before capture their swimbladders must have contained nitrogen at pressures as high as 9 atm.

A notable confirmation that it was insufficient to think only in terms of oxygen secretion came only a few years later when Schloesing and Richard (1898) made some very careful analyses of the gas obtained from deep-sea eels of the species *Synaphobranchus pinnatus* which had been caught at 900 and 1385 m depth. We may take as an example of their results the percentage composition of the gas in a specimen caught at 900 m. This was oxygen 75·1 per cent, nitrogen 20·5 per cent, carbon dioxide 3·1 per cent; the argon was 0·4 per cent, i.e. 1·94 per cent of the nitrogen as against 1·184 per cent for air. These authors stressed the difficulties of the measurement of argon. Let us assume that these results obtained at the surface represent the composition of the swimbladder gas just *before* the fish was caught. Since, to balance the hydrostatic pressures at 900 m depth, the total gas pressure must then have been about 90 atm, the approximate partial pressures of the individual gases would have been oxygen 68 atm, nitrogen 18 atm, carbon dioxide 3 atm and argon about $\frac{1}{3}$ atm. Now, in the sea the combined partial pressures of all these gases equals only about 1 atm* and, if we suppose that the fish had secreted these gases into its swimbladder when living at 900 m, we would be faced with the secretion, not only of oxygen, but also of nitrogen, carbon dioxide and argon against high gradients.

Before we take up the formidable task of explaining the simultaneous secretion of four gases which are so very different in properties, we can raise two objections to the above interpretation of Schloesing and Richard's results. First, there may have been some change in the composition of the swimbladder gas on hauling the fish from the bottom of the sea to the surface; carbon dioxide may well increase

* In the sea nitrogen is everywhere about 0·8 atm, oxygen is generally close to 0·2 atm but sometimes well below this figure, carbon dioxide around 0·001 and argon 0·01 atm.

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after death, and, if there were some exchange of gases with either sea or air during the haul the proportions of nitrogen and argon would increase at the expense of oxygen. Secondly, we have no assurance that the fish was in buoyancy equilibrium at 900 m; the swimbladder may have been smaller than that required for equilibrium and contained only gas secreted earlier when the fish was at lesser depth.

In the last few years new experiments have both confirmed and clarified this earlier work and given us very good reasons for accepting its implications. Scholander and van Dam (1953) have studied gas taken from the swimbladders of twenty-six species of bottom fish taken between 200 and 900 m. Bottom fish were chosen because it is easier to know the exact depth of capture and because such fish are less prone to make vertical migrations. To avoid changes in gas composition during hauling, this was made at great speed; 3–5 min for line fish caught between 300–420 m and less than 30 min for fish caught in an otter trawl. Since the composition of swimbladder gas in moribund fish lying on deck changed little in the hour or two following their coming in-board, Scholander and van Dam concluded that the gas composition had not changed appreciably during the quick haul from the bottom of the sea. Moreover, they found with individual species that both oxygen and nitrogen partial pressures rose almost linearly with the depth at which the fish had been caught (Fig. 5), a regularity unlikely if either of the two objections raised against the simple interpretation of Schloesing and Richard's results were valid ones. Scholander (1954) extended these experiments both to fishes living down to 1400 m and to include analyses for argon. Again, although oxygen was always the principal gas found, the partial pressure of nitrogen increased linearly with depth, the rate of increase differing in different species (Fig. 5). The values for the argon/nitrogen ratio lay between $0{\cdot}87 \times 10^{-2}$ and $1{\cdot}61 \times 10^{-2}$ and were therefore reasonably close to the $1{\cdot}18 \times 10^{-2}$ ratio found for air.

A general picture applying to the majority of fish may now be given. If fish live near the surface their swimbladders will contain a gas mixture not very dissimilar from air (approx. 80 per cent nitrogen, 20 per cent oxygen). The deeper the fish lives in the ocean the greater does the percentage of oxygen become; it often accounts for over 90 per cent of the total gas. The *fraction* of nitrogen falls with depth but its partial pressure rises from 0·8 atm at the surface sometimes to over 10 atm.

The coregonids studied by Hüfner formed a notable exception to this general picture and they were therefore restudied by Saunders (1953) and by Scholander *et al.* (1956a). These workers fully confirmed the earlier results. Nitrogen was always the most prominent constituent

of the swimbladder gases of these fishes. In one coregonid caught at 100 m Scholander *et al.* found that at the surface the volume of the swimbladder was twelve times that required to give it neutral buoyancy, so that even if it had been taken back to 100 m it would still have been positively buoyant. The nitrogen and argon accounted for over 99 per cent of the gas and the argon/nitrogen ratio was again close to that of air. In all these experiments carbon dioxide was never found to be the major constituent but varied from a negligible amount to a few per

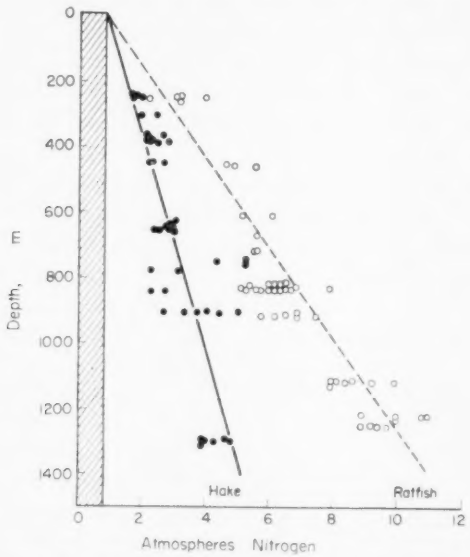

FIG. 5. Relation of partial pressure of nitrogen in the swimbladder to the depth at which the fish was caught. The shaded area is equal to the partial pressure of nitrogen in sea water. ○, *Macrurus bairdii* (the common ratfish). ●, Urophycis chesteri (the long finned hake). (After Scholander, 1954.)

It may be noted for example, that even in the ratfish at 1200 m, the partial pressure of nitrogen of about 10 atm will only balance about one-twelfth of the external pressure whilst oxygen will balance about eleven-twelfths of the external pressure.

cent. From the constancy of the ratio of nitrogen/argon found and from the closeness of this ratio to that found for air, the important conclusion has been drawn that nitrogen and argon are secreted by the same mechanism. Since argon takes part in virtually no chemical reactions it follows that they both must be secreted by some physical process.

Recently, Sundes (1959) has shown that the swimbladders of individual fish of the species *Coregonus laveratus* and *C. acronius* may contain much more oxygen than is usually found there.

When a fish has been for some time at a given depth the composition of the swimbladder gas will not be the same as that secreted by the gas gland. It will be the resultant of secretion followed by a later reabsorption and exchange of gases between the swimbladder and the surrounding tissues. To test theories of secretion the composition of the *secreted* gas is clearly of very great importance. We should particularly like to have this information for the deep-sea fish but, although they furnish us with spectacular demonstrations of the capacity of the swimbladder to produce and retain gases, they are barely accessible for experimentation. Work on newly secreted gas has only been made on fishes living in shallow waters.

Moreau induced fish to secrete gases in several quite different ways. He extracted gas from the swimbladders of physostomes by placing them under reduced pressures. The swimbladder gas then increased greatly in volume and was largely lost through the pneumatic duct. He extracted gas from physoclists by direct puncture of the swimbladders. He placed fish which had been in equilibrium at the surface in baskets lowered to 7–8 m depth. He attached small weights to fish. These quite different methods of rendering fish effectively denser than the surrounding sea water were all successful in persuading them to secrete more gas to restore buoyancy equilibrium. Moreau made experiments on a variety of fishes including perch, Moray eels, carp and wrasse, some of which are physostomes and others physoclists, and in all these the new gas secreted was overwhelmingly oxygen. After an initial secretion of gas and the restoration of neutral buoyancy most of the oxygen was slowly replaced by nitrogen. This discovery coupled with Biot's findings on deep-living fish fixed attention on oxygen as the secreted gas. The origin of this oxygen has recently been the subject of two most interesting pieces of work. Scholander *et al.* (1956b) added $^{18}O_2$ to the sea water surrounding fish which were actively secreting gas and later found $^{18}O_2$ in the swimbladders in amounts which showed the secreted oxygen to be derived entirely from that dissolved in the surrounding water and not from oxygen in chemical combination in sea water or the animals' tissues. Wittenberg (1960b), using a similar method, has shown that if molecules of $^{18}O_2$ are added to the sea water they appear in the swimbladder without having exchanged atoms with $^{16}O_2$, thus oxygen is probably secreted as a molecule and is not split into its constituent atoms during secretion.

Looking at the array of evidence presented, what is most surprising is that such diverse gases as oxygen, nitrogen and argon can all be concentrated in swimbladders. Considerable efforts have therefore been made to explain the secretion of all these gases in terms of the active secretion of only one of them or in terms of carbon dioxide which, unlike

the others, arises metabolically. We may with profit briefly examine the theories about, and the evidence for a role for carbon dioxide in gas secretion. Haldane's (1922) theory which is based on the known properties of fish blood is easy to understand and is accessible to experimental test. In it the local carbon dioxide concentration at the gas gland is increased and displaces oxygen from oxyhaemoglobin. A similar reaction is known to play an important role in the transport of oxygen in mammals where carbon dioxide produced by tissue metabolism diffuses into the capillaries and displaces oxygen from the oxyhaemoglobin of the arterial blood. This important reaction is known as the Bohr effect (see e.g. Henderson, 1928); the shape of the oxygen dissociation curve of mammalian blood being changed by carbon dioxide in such a way that an increase in carbon dioxide concentration will tend to displace oxygen from oxyhaemoglobin. In mammalian blood carbon dioxide or acidity has very little effect on the amount of oxygen which the blood will take up when the partial pressure of oxygen is high. Root (1931) and Green and Root (1933) found a related, but much more striking, reaction in fish blood in which not only the dissociation curve but the oxygen capacity of the blood is greatly changed by carbon dioxide or acidity. Fig. 6(a) shows, after Scholander and van Dam (1954), the effect of changes of carbon dioxide and acidity on the oxygen dissociation curve of the black grouper fish. In the blood of this fish a change in carbon dioxide from 5 per cent to 10 per cent, or a corresponding change in acidity, will displace more than half the oxygen combined as oxyhaemoglobin even against an oxygen pressure of 80 (or even 140) atm.

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To show that this mechanism plays some role in the secretion of oxygen it would seem adequate to find an appreciable increase in acidity and carbon-dioxide in the gas gland. Hall (1924) dialysed the gas glands of the yellow perch and showed that when secretion was stimulated by removing gas from the swimbladder the dialystate's *p*H fell from 7·1 to 6·4 in 45 min. Jacobs (1932) and Akita (1936) found that when gas was withdrawn from a swimbladder the newly secreted gas was at first rich in carbon dioxide. The course of such secretion was also studied by Fänge (1953) and a curve taken from his results is shown in Fig. 7. In this figure it may be seen that, although there is an appreciable amount of carbon dioxide in the secreted gas, the rate of secretion of oxygen at all times is much greater than that of carbon dioxide. Scholander (1956), using the very elegant microgasometric techniques which he and his colleagues have developed, has analysed the gas secreted as bubbles on the inside of the swimbladder by an active gas gland. He found, for the cod, that this contained 5–16 per cent carbon dioxide and 30–81 per cent oxygen.

According to Haldane's view the rete would allow a high concentration of carbon dioxide and acidity to be built up by preventing carbon dioxide from escaping from the site of its production in the gas gland. This might be done either by the simple diffusion or by the active secretion of carbon dioxide from the venous to the arterial capillaries of the rete. This hypothesis was extended by Powers (1932) to explain the accumulation of nitrogen and argon. On his theory the combined

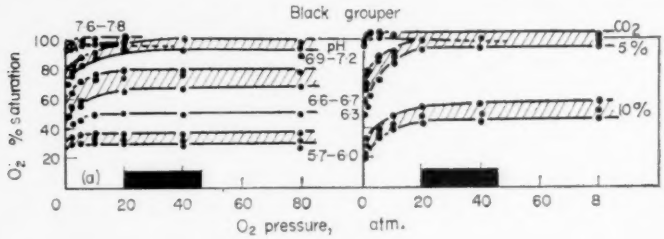
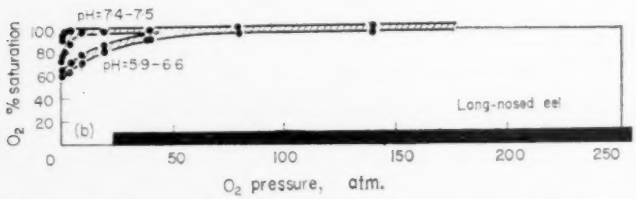


FIG. 6(a). Black grouper fish. Oxygen dissociation curves at 10°C. Lactic acid was added to the blood on the left, CO_2 to that on the right. The figures alongside the curves on the left are of pH. The blood of the fish shows a strong "Root effect" even at pressures of 80 atm of oxygen, the addition of lactic acid or CO_2 displaces oxygen from oxyhaemoglobin.



(b). Long-nosed eel. Oxygen dissociation curves at 4°C. Lactic acid has been added to give, after aeration, the pH stated. Above 50 atm no "Root" effect is shown. The solid bars on the base lines show the depth range at which the fish live, Scholander and van Dam (1954).

partial pressures of carbon dioxide and oxygen are made to exceed the external pressure and bubbles containing these gases are formed. Nitrogen and argon will then diffuse into these bubbles up to a possible partial pressure of 0·8 atm for nitrogen and 0·01 atm for argon, i.e. their partial pressures in sea water. Such bubbles can then be discharged into the swimbladder carrying nitrogen and argon with them even if the partial pressures of nitrogen and argon in the swimbladder exceed those in the bubbles. A later preferential reabsorption of

oxygen and carbon dioxide might leave overwhelmingly nitrogen and argon behind.

Although on such relatively simple bases we could plausibly explain gas secretion in some fishes, these hypotheses are not adequate for all circumstances. Scholander (1954) has shown that in some deep-sea fish the action of carbon dioxide on oxyhaemoglobin could not produce oxygen against the very high partial pressures of oxygen which are known to exist in their swimbladders. Fig. 6(b) shows, for example, that

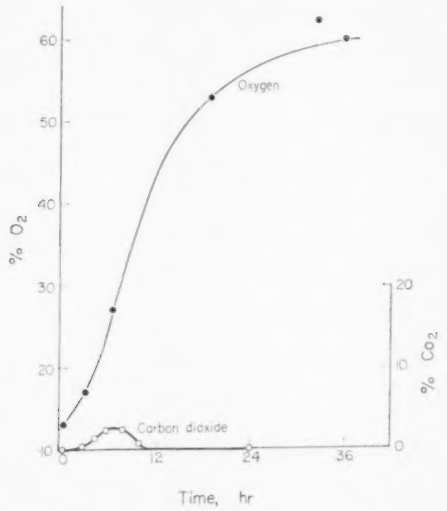


FIG. 7. The composition of gas in the swimbladder of *Ctenolabrus rupestris*. Gas has been removed from the swimbladder by puncture and new gas is being secreted by the gas gland. The oxygen initially secreted would only slowly be replaced by nitrogen; it would be several weeks before the oxygen fell to its usual value of about 14 per cent. (After Fänge, 1953.)

at high pressures of oxygen there is no Root effect in the blood of the long-nosed eel. We must postulate, therefore, some mechanism, other than that suggested by Haldane, to explain gas secretion at great depth in these fish. This does not, of course, prove that the Bohr and Root effects may not be used for gas secretion in these fish at lesser depths and in other fish at all depths; indeed there is no compelling theoretical reason for believing that one mechanism of secretion is universally used. Nevertheless, we are reluctant to admit that the unknown mechanism, which we must postulate for secretion against high pressures, is not also the one used for secretion against low pressures.

Recently Wittenberg and Wittenberg (1960) have thrown doubt on the adequacy of Haldane's hypothesis even for fish secreting gas against low pressures. They brought sea-water into equilibrium with gas mixtures containing 50 per cent oxygen and varying amount of carbon monoxide. Into such sea waters they placed toadfish (*Opsanus tau* L.) from which the swimbladder gas had just been removed, and made the striking observation that the gas which they secreted not only contained carbon monoxide but generally contained it in concentrations from seven to nine times higher than did the original gas mixtures. In the limiting conditions which they studied the gas mixtures contained 0·19 per cent and 34 per cent carbon monoxide whilst the secreted gases contained 1·3 per cent and 50 per cent carbon monoxide, respectively. In these experiments the ratio of

$$\frac{\text{partial pressure of CO secreted}}{\text{p.p. CO in sea-water}} \div \frac{\text{p.p. } O_2 \text{ secreted}}{\text{p.p. } O_2 \text{ in sea-water}}$$

was found to remain fairly constant at about 5.

The Wittenbergs argue that this constancy would be very unlikely if secretion depended on the expulsion of carbon monoxide and oxygen from blood haemoglobin by acid or carbon dioxide. Moreover in some toadfish they have found that even when 97 per cent of the circulating haemoglobin was in the carboxy form, the secreted gas contained 35 per cent oxygen. They consider that carbon monoxide and oxygen compete for a common intracellular carrier, perhaps an intracellular haemoglobin.

Wittenberg (1958) has made another notable contribution to our knowledge of the swimbladder. In a number of species he has measured the concentrations of nitrogen, argon, neon and helium in *freshly secreted* gas. The fish were in shallow water and the total pressure of gas within the swimbladder was always close to 1 atm. The secretion of gas was induced by removing gas from the swimbladder by puncture. We may recall that in these conditions the newly secreted gas is usually principally oxygen and that, after secretion is complete, the fraction of nitrogen (and argon) rises slowly until finally nitrogen accounts for most of the swimbladder gas. Wittenburg found for five species (goldfish, toadfish, a fresh-water eel and two species of sea robin) that, although the partial pressures of nitrogen and argon were low in freshly secreted gas, the ratio of argon to nitrogen was appreciably higher than in air. The ratio in air is $1{\cdot}18 \times 10^{-2}$ whilst the highest values of argon to nitrogen in newly secreted gas averaged $2{\cdot}33 \times 10^{-2}$. Since argon is about twice as soluble as nitrogen in water this latter value approaches the ratio $2{\cdot}64 \times 10^{-2}$ for the gases *dissolved* in

air-saturated water. The accumulation of neon and helium from sea water equilibrated with gas mixtures containing these gases was also studied. Helium and neon, which are both less soluble in water than nitrogen, were found in lower concentration in the secreted gas than in the gas mixtures. The ratios of nitrogen, argon, helium and neon in freshly secreted gas approached those which would be given if secretion were accompanied by the complete exhaustion of these gases from a given volume of water. Wittenberg explains this by assuming that oxygen is actively secreted in the form of minute bubbles into which inert gases and nitrogen diffuse. These bubbles which are principally of oxygen but contain a slight proportion of other gases then pass into the swimbladder. Now a gas space in equilibrium with water will hold a very much larger mass of gas per unit volume than does the water. A small bubble of oxygen will act as a reservoir into which other gases will diffuse until the "shell" of water immediately around the bubble is almost exhausted of these gases which will finally be present in low partial pressure in both bubble and surrounding water.

The five species listed above gave results which were in good accord with Wittenberg's hypothesis whilst three others did not. The scup (*Stenotomus versicoler* Mitchell) which replaced gas taken from the swimbladder in 12 hr and secreted gas more quickly than any other species studied, gave lower argon to nitrogen ratios than expected. In two species of trout the secreted gas consisted of 95–99 per cent nitrogen and the argon to nitrogen ratio was the same as that of air. Wittenberg believes that a transient secretion of oxygen occurs in the trout but was not demonstrable because oxygen removal from the swimbladder was too rapid relative to the rate of secretion. He also argues that gas secretion cannot depend on an initial secretion of carbon dioxide bubbles into which oxygen and other gases diffuse for, since the oxygen content of tissue fluids is only about half that of nitrogen, the ratio oxygen to nitrogen in the swimbladder could not exceed 1 : 2 whilst ratios of over 2 : 1 have commonly been observed.

Wittenberg's hypothesis is interesting but another explanation of the experimental results may be given—this depends on the function of the rete and will be discussed after a short consideration of this remarkable organ.

1.5. *The rete mirabile.* Since the elucidation of the structure of the rete mirabile its possible role in gas secretion has continued to excite curiosity and calculation.

This role must be an important one, for although it is true that some fish which are thought to lack a rete can secrete gases, they can only do so at a low rate. Fish living deep in the ocean which have to secrete gases against very great pressures always have a very well-developed

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rete mirabile. Marshall (1960) has made a notable study of the swimbladders of these animals and the following account is drawn from his work. The great majority of species have a unipolar rete like that of the perch (Fig. 1b); sometimes this is a single body but often it is split into a number of smaller units going to different areas of the gas gland. Only the deep-sea eels and stomiatoid fishes have bipolar retes like that of

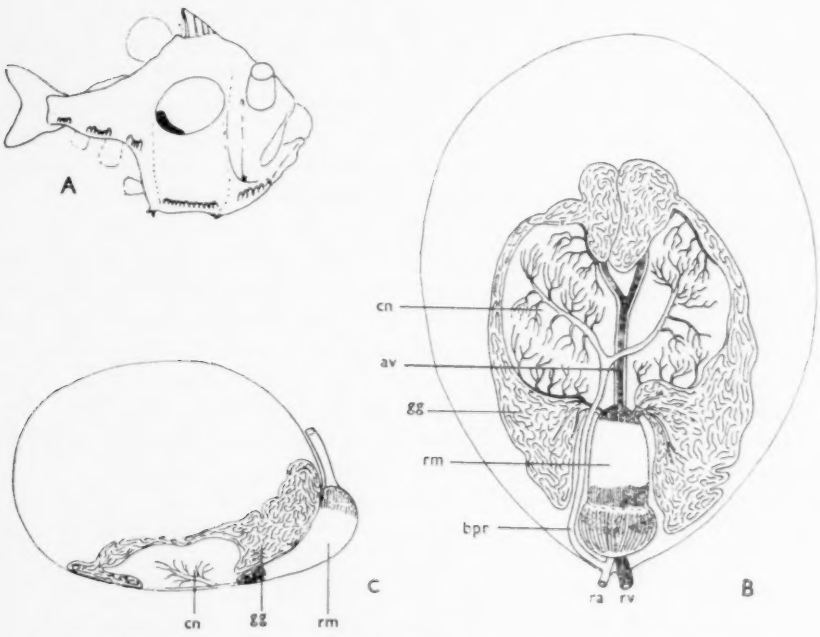

FIG. 8. Diagram of a stomiatoid fish with an artery which bypasses the rete mirabile. *A*, *Argyropelecus aculeatus*, *B*, its swimbladder, ventral view, *C*, its swimbladder, lateral view. g.g. gas gland; r.m. rete mirabile; b.p.r. by-pass branch of retial artery; r.a. retial artery; r.v. retial vein; c.n. capillary network; a.v. artery vein pair. (After Marshall, 1960.)

Anguilla (Fig. 1a). The swimbladder wall of the stomiatoid has no region like the oval of the perch, with a circulation completely independent of the rete, but instead an artery which by-passes the rete, with a venous return through the rete (Fig. 8). By the opening of this artery the swimbladder can presumably be served by arterial blood which does not go through the counter-current diffusion system of the rete and which can therefore be used to carry away gases which the fish wishes to reabsorb.

Whatever the anatomical details all the deep-sea fish have an extreme development of gas gland and reabsorbing regions. These

cover a much greater fraction of the swimbladder wall than in the surface fish. The ratio

$$\frac{\text{surface area of gas gland (mm}^2\text{)}}{\text{volume of swimbladder (mm}^3\text{)}}$$

in the surface forms lies usually between $\frac{1}{25}$ and $\frac{1}{160}$, in the bathypelagic species it is between $\frac{1}{2}$ and $\frac{1}{16}$. The rete also becomes longer with increasing depth of habitat; thus the myctophids and stomiatoids centred around depth of 200–1000 m have retia between 0·75 and 4·0 mm long whilst in the benthic species they generally are between 7 and 13 mm long. The rete reaches its extreme development in the swimbladders of the abyssal fishes. Thus Marshall describes in *Lionurus filicauda* six retia each of 20 mm length and in *Bassozetus taenia* 2 retia each 25 mm long.

One of the greatest gaps in our knowledge of the swimbladder is that of the gas composition of the blood at various parts of the rete and gas gland during the active secretion of gas. We have to assume that the rete has an important role in the secretion and retention of gases in the swimbladder and think how it might work. It is evidently designed to enable exchanges of substances to take place between its arterial and venous capillaries. These substances may exchange either by diffusion or by secretion and we will consider these two possibilities.

If all the blood to the gas gland passes through the rete we can show the arrangement on a simple diagram (Fig. 9). Even if there is an exchange of liquid across the rete the volume of blood entering A must over any appreciable length of time equal that leaving at E; similarly the volume of blood leaving B must equal that returning to D. Even if the rete's capillaries have merely the common properties of capillaries with respect to the diffusion of gases, it is easy to see how the rete could help to *maintain* a high concentration of a gas if this were once built up in the swimbladder and in the region BCD. As an example let us take argon or nitrogen, for these are the gases least likely to be secreted and most likely to have their distribution determined by simple diffusion. Taking possible values for a deep-sea fish, the partial pressures in the swimbladder and in the blood leaving the gas gland might be 15 atm for nitrogen and 0·2 atm for argon whilst in the blood entering at A the partial pressures will be approximately 0·8 atm for nitrogen and 0·01 atm for argon. The nitrogen and argon will diffuse from the outgoing stream into the ingoing stream (Fig. 9) and finally, the blood stream leaving at E will contain nitrogen and argon at a concentration only a little higher than in the entrant stream at A.

The simple system just described is admirable for the retention of

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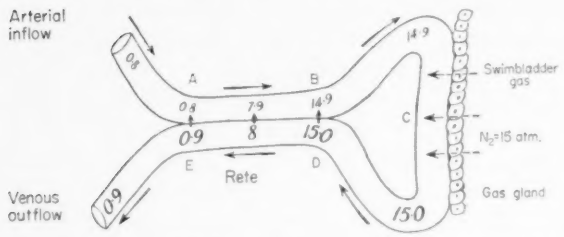

FIG. 9. Diagram of a swimbladder with a rete mirabile. This shows how the counter-current diffusion system in the rete could very much reduce the loss of a gas from the swimbladder once this gas has been secreted. The arrows outside the vessels show the direction of blood flow. Those inside the direction in which the gas, here nitrogen, moves. The figures are partial pressures in atmospheres.

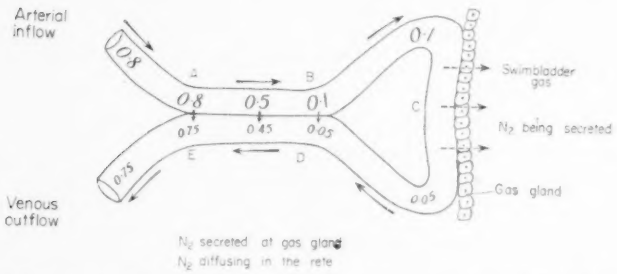

FIG. 10. Similar to Fig. 9 but showing how the rete mirabile might slow down the rate at which gas reaches the gas gland once some of the gas has been carried into the swimbladder.

gases but it would be fatal for their secretion, for the rete would make it just as hard to get gases into the swimbladder as it makes it hard for them to escape. The secretion of any gas demands that the blood leaving *E* contains it in lower concentration than that entering at *A* and this is exactly what the simple counter-current system we have described will prevent. Fig. 10 shows what would happen if the gas gland had lost nitrogen to the swimbladder so reducing its partial pressure in the blood surrounding the gas gland. The counter-current system of the rete will tend to stop more nitrogen reaching the gas gland.

At a given partial pressure of nitrogen the amount dissolved in blood is simply the small amount dissolved in physical solution and this is proportional to the partial pressure of nitrogen. If there is a difference in partial pressure of nitrogen between the two ends of the rete then both gas content and partial pressure will change linearly with distance between these two ends. If the partial pressure of nitrogen at one end

of a retial capillary is 0·8 atm and at the other end 0·2 atm then at a point half-way between the ends the partial pressure of nitrogen will be 0·5 atm. Such a simple relationship will also be found for oxygen when the partial pressures of oxygen are everywhere high and the blood haemoglobin everywhere saturated with oxygen. At lower partial pressures, when haemoglobin is not fully saturated with oxygen, there can be no such simple relationship between distance along the rete and the blood's content of oxygen. Since the content of oxygen is high and changes greatly for a small change in its partial pressure the rete might be much less effective at preventing oxygen from entering or leaving the gas gland than it is at holding back nitrogen. However, the retial capillaries are so long and the area for diffusion so large that the rete might be expected to act as an efficient counter-current diffusion system even for oxygen.

If the only method of exchange of a gas in the rete is to be diffusion then an efficient secretion of gas into the swimbladder by the gas gland might be obtained:

(1) If, during active secretion the secreted gas cannot diffuse between the two blood streams in the rete. This would be an absurd system since it would provide a leak of gas from the swimbladder just when secretion was trying to push gas into it.

(2) If there was a change in solubility of gases in the region *BCD*, (Fig. 9) which diffusion across the rete did not abolish. The blood could then leave *E* with the gases exerting almost the same partial pressure as at *A* but being in lower concentration. All gases might then be secreted by one mechanism. This hypothesis was suggested by Koch (1934). The difficulty in formulating it fully is that of finding a plausible method of decreasing solubility. To maintain an appreciable difference in temperature between incoming and outgoing capillaries in the rete is impossible whilst Scholander (1954) has calculated that an increase in solute content in the gas gland which would decrease the solubility of nitrogen by 1 per cent could provide a secretion of this gas at a 26 atm pressure although only at a very slow rate.

(3) If carbon dioxide which is the only swimbladder gas to arise metabolically in the fish were produced between *B* and *D*. The counter-current system of the rete would allow the carbon dioxide produced to be retained in this region and the pressure of carbon dioxide to rise. The carbon dioxide would scarcely be likely to arise by aerobic reactions since it would then be produced at the expense of oxygen and the counter-current system would then stop more oxygen getting to the gas gland. The production of an acid at *D* (say lactic acid) anaerobically would be more rational. This could raise the partial pressure of carbon dioxide and hence the oxygen partial pressure and if the acid were

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not free to diffuse across the retial capillaries it could allow the blood to leave *E* containing less oxygen and carbon dioxide than when it entered at *A*. The accumulation of oxygen and carbon dioxide would be limited by the maximum possible change in acidity which could be produced with such a constant loss of the acid. On this view the secretion of nitrogen and argon could take place by the method suggested by Powers, but would be very slow because of the counter-current system of the rete (Fig. 10). Nitrogen and argon are, however, secreted very slowly and this is not a fatal objection to this hypothesis.

My colleague Dr. T. I. Shaw has pointed out to me that at the temperatures often found in the ocean carbon dioxide would be liquified when its partial pressure rose above 45 atm, so that in deep-sea fish living below 450 m there can be no question of almost pure carbon dioxide bubbles being formed into which other gases diffuse.

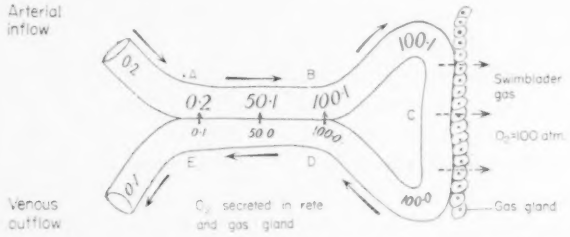

FIG. 11. Similar to Fig. 9 but showing how an O_2 secreting mechanism which can only sustain a small pressure difference at one point could be used in the rete to produce an enormous pressure of oxygen at the gas gland.

If the rete, instead of being completely passive, can actively secrete gases, it is easy to account for the secretion of any particular gas by merely assuming that this gas is secreted not only across the gas gland but actively from the venous to the arterial rete capillaries. Scholander (1958) has noted that the counter-current flow of the rete would allow a mechanism which could only sustain a small pressure difference between arterial and venous flows at any one point, to give rise over the whole length of the rete to a formidable pressure difference between the arterial blood coming to the rete and that leaving the rete to enter the gas gland (Fig. 11). Haldane suggested such a secretion of carbon dioxide and Wittenberg and Wittenberg (1960) argue that a secretion of oxygen from the venous to the arterial capillaries takes place. The extra length of rete found in deep-sea fish would then exist not primarily for the retention of gas, but to allow a large pressure of oxygen to be built up, by what has been called a counter-current multiplication system.

If only oxygen were secreted the counter diffusion system of the rete would still act as a barrier to nitrogen and argon and carbon dioxide. The carbon-dioxide often found in fair concentration during the initial secretion might then be merely that produced metabolically in the reactions which provide the energy for the secretion of oxygen.

With this discussion of the possible functions of the rete in mind we may re-examine Wittenberg's important evidence on the secretion of nitrogen and the inert gases. Let us suppose that there is a mechanism capable of secreting oxygen (and/or carbon dioxide) into the swimbladder. When, for example, gas has been taken from the swimbladder then the secreting mechanism becomes active and a volume of newly secreted oxygen (and/or carbon dioxide) is produced as a froth of bubbles over the gas gland. Since this secreted gas will contain other gases at much lower partial pressure than does blood these gases will diffuse from the blood circulating in the gas gland into the swimbladder, their partial pressures in this blood will fall and because of the rete will then be held at low levels (Fig. 10). The ratios of these gases *immediately* lost to the swimbladder will be approximately proportioned to the previous blood *content* of these gases (this agrees with Wittenberg's finding). Nitrogen and argon and other inert gases will now slowly enter the region *BCD* against the rete's counter-diffusion barrier. Blood will enter the rete with a content of these gases approximately equal to that in the external sea water but will leave the rete to go to the gas gland with a very low content of these gases. Under these conditions if two gases have approximately the same diffusion constant then an approximately constant fraction of each will cross the rete to reach the gas gland and so be available for "secretion" into the swimbladder. Argon and nitrogen would continue to be secreted in proportion to their *content* in blood and tissue fluids. In the freshly secreted gas neon and helium would enter the swimbladder less easily than argon both because they are less soluble in body fluids and because they probably have a higher diffusion constant.

This modified form of Wittenberg's hypothesis would explain why gas within the swimbladder comes to equilibrium with respect to helium and neon at "unexpectedly slow rates". This slowness of penetration would merely represent the effectiveness of the rete as a diffusion barrier to the penetration of diffusible gases (Fig. 10). This hypothesis differs from Wittenberg's in that it seeks the explanation of the experimental results on the secretion of nitrogen and the inert gases in terms of the properties of the rete rather than in local effects at the gas gland. It would not lead to the certain conclusion that oxygen is the *secreted* gas. The large fraction of oxygen found in the gas secreted would merely reflect the large oxygen content of blood.

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The possibility also remains open that gases may be secreted by a method which depends on changes in the general solubility of gas or by some other physical mechanism by which gases are secreted in amounts depending approximately on their availability in the blood.

1.6. *Summary.* We may then give a brief summary of the present state of our knowledge of gas secretion. Some fish in which the rete mirabile is either absent or not very evident can secrete gases at a relatively slow rate against pressures of up to 12 atm. This gas is generally found to be almost pure nitrogen even in gas which has recently been secreted into the swimbladder. Nitrogen is not secreted in preference to argon and, since argon is inert chemically, it is thought that both are secreted by some common "physical" process. It is assumed by some authors that in these fish oxygen is secreted carrying other gases with it and that oxygen once secreted is then quickly reabsorbed, leaving the other gases behind in the swimbladder. If a fish has no rete then the whole of the gas-pressure difference between swimbladder and blood must be produced and maintained by and across the cells of the gas gland. It is not known how cells can do this.

In fish which have a well-developed and obvious rete mirabile the gas *secreted* is principally oxygen derived from that dissolved in the surrounding sea water. It is secreted as molecules of oxygen which are not broken apart in the process of secretion, a finding which puts severe restrictions on any possible carrier mechanism which might be postulated for oxygen transport. Nitrogen and argon are again thought to be secreted by some common path. Carbon dioxide is usually present in very small amounts in the swimbladder but the amount rises when active secretion takes place. Although, for surface fishes, plausible theories have been advanced to explain the secretion of other gases in terms of an initial secretion of carbon dioxide, in some deep-sea fish carbon dioxide cannot be the gas primarily secreted. Now, although we ought not to close our eyes to the possibility that more than one mechanism of secretion has been evolved, we must hope to find a simple explanation of secretion which will fit all cases and, for the present, reject theories based exclusively on a carbon dioxide secretion.

If one mechanism is to fit everywhere there would seem to be two possibilities. First, that oxygen is secreted and that other gases are carried with it into the swimbladder, second, that all the gases are secreted by some "physical" mechanism, more oxygen being secreted simply because the content of oxygen of blood is so high. In deep-sea fish it seems almost essential to assume that the rete mirabile plays an important role other than as a counter-diffusion barrier and that in it a gas or gases are secreted from the venous

to the arterial capillaries. If this secretion does not take place then blood leaving the rete *must* differ from that entering the rete in that gases are less soluble in it. Again we have a choice of hypotheses between the secretion of all gases or the secretion merely of oxygen.

In conclusion we may say that the very excellent work of recent years has given us a number of very useful criteria on which theories of secretion may be judged but no theory so far advanced can fully explain the secretion of all gases in all fishes and, indeed, we have not yet a hypothesis fully adequate to account for the secretion of gas in a single species.

2. *Fish without a gas-filled swimbladder*

We will leave aside the common coastal bottom fish which have not and need no gas-filled swimbladder. Of the pelagic fish, most of the teleosts have effective swimbladders but some, including the mackerel and some species of tunny, have none and are denser than sea water. When a mackerel is watched in an aquarium it can be seen to maintain its level in mid water only by restless and vigorous activity; if it slows down it sinks. This unceasing motion is, perhaps, the price paid for facility in moving up and down quickly in the top layers of the sea; for to come quickly to the surface from 20 m would increase the volume of the swimbladder threefold, and, apart from the danger of internal damage, the fish would have to exert a force equal to about 10 per cent of its weight in order to go down again.

In some of the mid-water pelagic oceanic fish, the swimbladder contains fat and no gas. A swimbladder filled with fat of density about 0·9 will only be about one-tenth as useful in giving buoyancy as one containing an equal volume of gas. Some fish appear, however, to have large amounts of fat, thus *Cyclothone*, one of the world's most common fish, has in addition to a fat-filled swimbladder, fat deposited in the mesenteries and a well-developed system of fat sinuses under the skin. All these fat reserves taken together may occupy 15 per cent of the body volume (Marshall, 1960) and such a fish may not be far from neutral buoyancy even although it has no gas-filled swimbladder.

Many other mid-water fish, for example the well-publicized, deep-sea angler fishes have no such high content of fat and yet seem singularly ill-constructed for swimming hard continuously. Two such bathypelagic fish without a gas-filled swimbladder were studied by Denton and Marshall (1958). One of these, *Gonostoma elongatum*, had a weight in sea water of only about $\frac{1}{2}$ per cent of its weight in air whilst the other *Xenodermicthys copei* was about 1·2 per cent denser than sea water. (Deprived of their gas-filled swimbladder most common pelagic fish would be about 5 per cent denser than sea water.) The fat content of

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these deep-sea fishes (less than 3 per cent) was not particularly high and could not therefore account for their buoyancy. This was explained by the demonstration that the components which principally "sink" a fish, i.e. the muscles and the minerals of the skeleton, are present only in

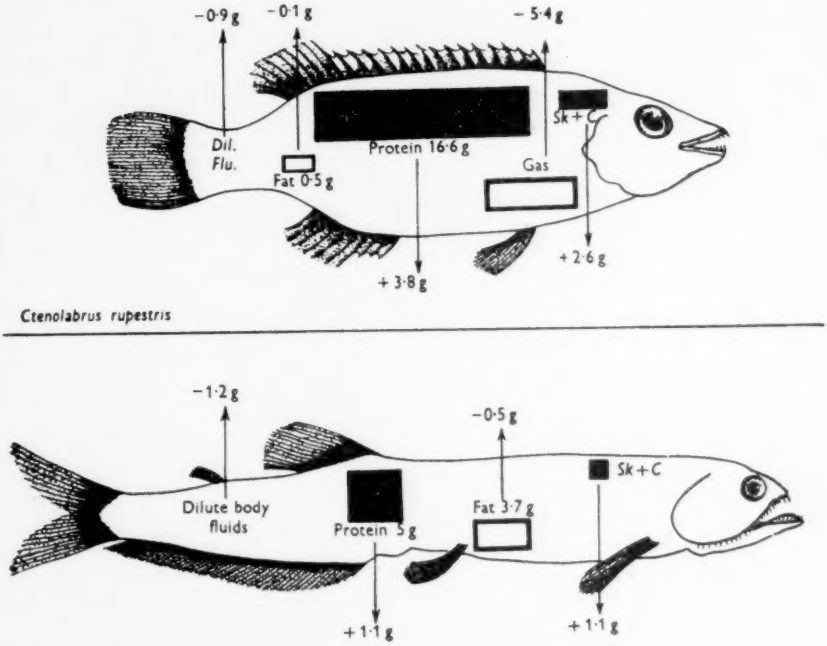

FIG. 12. Diagram of the "buoyancy balance sheet" for a bathypelagic fish, *Gonostoma elongatum* without a swimbladder (below) and a coastal fish *Ctenolabrus rupestris* with a swimbladder (above). Positive values are given for those components of the fish which are heavier than the sea water which they displace and thus tend to "sink" the fish, whilst negative values are given for those components which displace more sea water than their own weight and thus tend to "float" the fish. Weights given per 100 g of fish. *Dil. Flu.*, dilute body fluids; *Sk + C*, skeleton and other components. (After Denton and Marshall, 1958.)

small amounts. Such a fish will have about 5 per cent protein whilst a typical coastal fish will have about 17 per cent protein (Fig. 12). The skeleton on which the attenuated muscles pull is very slight as the radiograph on Plate I shows. These fish resemble other fish in containing fat and dilute body fluids (which will tend to give them positive buoyancy) so that they can approach neutral buoyancy without a gas-filled swimbladder.

Bathypelagic fish without a swimbladder are quite diverse, accounting for nearly half of the bathypelagic fish fauna (Marshall, 1955). They include not only fish of the large orders Isospondyli and Inioni but also the members of the small orders Giganturoidea, Lyomeri,

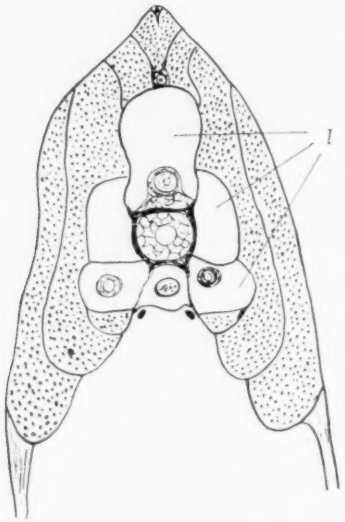

FIG. 13. Transverse section through the fore-part of the trunk of the gulper-eel, *Eurypharynx pelecanoides*, showing the extent of the muscle (dotted) and the lymphatic spaces (*l*). (After Nusbaum-Hilarowicz, 1923.)

Cetunculi and Miripinnati. In these fish the nature and extent of the tissues appear to be very similar to those which Denton and Marshall have analysed in detail (Bertelson, 1951; Marshall, 1955; Beebe and Crane, 1939; Tchernavin, 1947). The weak lateral muscles in one species, a gulper-fish (*Eurypharynx pelecanoides*) is well shown in a transverse section figured by Nusbaum-Hilarowicz (1923). The vertebrate column is surrounded by extensive fluid-filled cavities which appear to be lymphatic in nature (Fig. 13).

In evading, or almost evading, the buoyancy problem, it is the muscles used in propulsion that are particularly reduced. This might seem, because of loss of speed, to be like "jumping out of the frying pan into the fire". These fish are known, however, to catch and eat prey as large as themselves and we must ask ourselves how they can do this with such weak muscles. The answer seems to be that they live in the dark of the deeper ocean waters and attract their prey with

luminous lures. The amount of muscle which an animal must have depends upon the *peak* effort which it makes. Muscle which is useful only on rare occasions is, at all other times, a nuisance. These deep-sea fish, by luring their prey, avoid high peak efforts and make do with little muscle and a light skeleton. The muscle and skeleton systems are particularly reduced along the trunk and tail whilst the gill arches and the main structures for holding and swallowing their prey, have the strongest muscles and the most firmly ossified parts of the skeleton. These fishes are floating traps.

III. CEPHALOPODS

1. *Cephalopods without gas-filled buoyancy chambers*

1.1. *General notes.* The great majority of modern cephalopods have no gas-filled chambered shell like *Nautilus*, *Spirula* or *Sepia* (described in section 2). Some like the common octupus are bottom living and have no special buoyancy mechanism. Others, and these are the great majority, are pelagic, but the buoyancy of only a few of these has been studied.

Loligo forbesi, the common coastal squid is about 4 per cent denser than sea water and like the mackerel must swim all its life to avoid sinking. Its shell is reduced to the thin transparent "pen" which has no gas chambers. The pen has a weight of only 0·6 per cent of that of the whole animal and since its density is only about 1·2 it contributes very little to the "buoyancy balance sheet" of the squid (Denton and Gilpin-Brown, unpublished observation). The same figures are approximately true of the very common oceanic squid *Ommastrephes pteropus*.

Some of the deep-sea squid and octopods have very reduced musculature, ink sac and radula, and perhaps in buoyancy they resemble the deep-sea fish studied by Denton and Marshall (1958) (see p. 212). Many of them contain a large amount of gelatinous material but, since it is not known whether the density of this material is greater or less than that of sea water (Marshall, 1954), we can only leave the problem of their buoyancy to later workers.

1.2. *The cranchid squid.* A very remarkable buoyancy mechanism has been found in one family of oceanic cephalopods, the Cranchidae (Denton, *et al.*, 1958). The cranchids are a family of squid which are world-wide in distribution. Some members which have recently been caught second-hand (in the stomachs of sperm whales) are very large. One specimen of *Mesonychoteuthis hamiltoni* Robson studied by Dr. Anna Bidder was about 4 m long.

Three species of cranchid squid (*Verrilliteuthis hyperborea* Steenstrup, *Galiteuthis armata* Joubin and *Helicocranchia pfefferi* Massy) were studied alive aboard *R.V. Sarsia*. *Helicocranchia* was particularly commonly caught. In a sea-water tank aboard ship they were found to be able to hang almost motionless head downwards without effort (Mr. P. M. David of the National Institute of Oceanography tells me that in his experience aboard *R.R.S. Discovery* they more commonly drifted upwards at a very slow rate). They can swim by using water jets from their siphon aided by two small fins at their stern.

Having no chambered shell like *Nautilus*, *Spirula* or *Sepia* and no air sac like a fish they must have some other component less dense than sea-water to balance the tendency of their proteins to sink them. If the mantle of a freshly-caught cranchid squid is opened by a cut along its ventral surface an enormous coelomic cavity filled with fluid can then be seen. This coelomic cavity which almost fills the mantle cavity is covered by a transparent membrane and through this membrane such organs as the stomach, caecum and reproductive gland can clearly be seen (Fig. 14). When this membrane was punctured and the fluid from within the coelomic cavity drained away, the squid sank. The buoyancy equilibrium of the intact animals had clearly depended on the low densities of their coelomic fluids. This fluid, which amounted to about two-thirds of the squid's total volume, was noted as being transparent, colourless and of bitter taste, and then stored in a deep freeze and studied later in a land laboratory. From the volumes of coelomic fluid, the weights of the "drained" animals under sea-water, and the density of that water, it was calculated that the densities of the coelomic fluids must have been close to 1·010 to give neutral buoyancy. Density bottle measurements showed that coelomic fluids from all the specimens of the three species studied had densities between 1·010 and 1·012. Such low densities could not be given simply by replacing the "heavier" ions of sea-water by the "lighter" ones, e.g. replacing calcium by sodium, and sulphate by chloride (p. 183). It was therefore suspected that the coelomic fluids were hypotonic to sea water. Measurement of freezing points showed, however, that these fluids were almost isotonic with sea-water. Of the common ions only ammonium can give a solution isotonic with sea-water but of density close to 1·010. Ammonia was shown to be present in fair concentration by adding caustic soda to the coelomic fluid when a strong smell of ammonia was observed. The concentration of ammonia was estimated by the method of Crowther and Large (1956) modified by F. A. J. Armstrong of the Plymouth laboratory. By this method, which is insensitive to other amines, the coelomic fluids were shown to contain about 480 mM ammonium. The sodium concentration was shown to be 90 mM whilst

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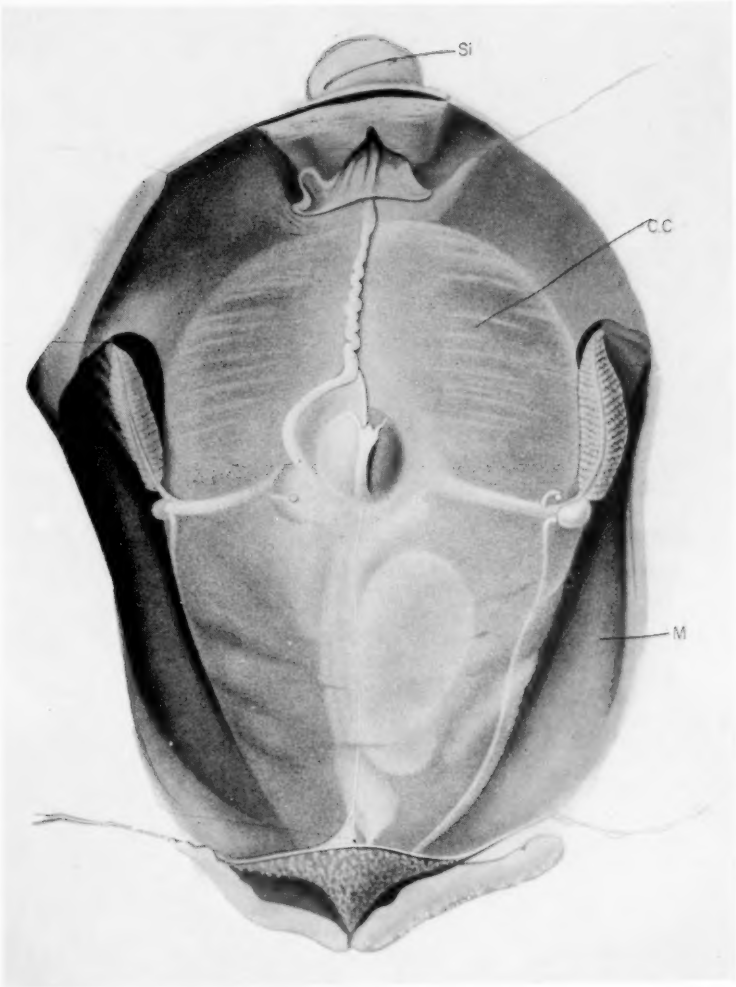

FIG. 14. *Cranchia scabra*. The mantle of the squid has been cut along the mid-ventral line and the large liquid-filled coelomic cavity can be seen. The head end of the animal is upwards. *M*, mantle; *C.C.*, coelomic cavity; Si, siphon. (After Chun, 1910.) Magnification $\times \frac{1}{3}$.

[*facing p. 212*

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chloride accounted for almost all the anion. The fluid was very acid, *p*H around 5, and perhaps this accounts for the retention of ammonia in the coelomic cavity. At this acidity ammonia would exist almost completely as ammonium, which in contrast to molecular ammonia does not pass easily through living tissues.

2. *Cephalopods with gas-filled buoyancy chambers*

2.1. *Fossil cephalopods.* The immensely numerous fossil shells of the early cephalopods—the nautiloids, ammonoids and belemnoids—have long furnished an ample and accessible material for reflection, deduction and speculation about the habits of their former owners and since these shells are almost all that remain to us problems of buoyancy have naturally attracted great interest. The very evolution of these molluscs, from a crawling to a free-swimming life was probably determined by a particular solution to the problem of buoyancy and we may perhaps digress a little to recapitulate after Morton (1958) a pleasing, plausible but uncertain evolutionary history.

The first nautiloids in the Upper Cambrian were the earliest cephalopods. We may imagine that they evolved gas-filled chambers at the apex of their cap-shaped shells which increased in importance until the animal could easily take off from the bottom using jets of water for locomotion. The foot, freed from the task of crawling, spread forward to surround the mouth, its edge became a fringe of tentacles and thus the distinctive cephalopod pattern emerged.

Buoyed by their gas-filled shells, the nautiloids, ammonoids and belemnoids were enabled to range and to dominate the Palaeozoic and Mesozoic seas. Many and beautiful are the forms which they tried for the chambered shell to which they owed their evolution and success, Buckland (1837). Some cephalopods had long straight "orthocones" (Fig. 15) and may perhaps have swum quickly, others had plump helmet-shaped shells "brevicones", and must have drifted head downwards, the animal peeping, respiring and pushing out arms through relatively small apertures at the base of the shell. To achieve particular postures the shells were sometimes bent, and sometimes coiled in plain spirals or helices; often they were differentially weighted by the secretion of solid masses of calcareous material (Figs. 15 and 16).

The 163 families of the Ammonoidea and the five families of Belemnoidea are extinct; of the seventy-five families of Nautiloidea only one genus, the famous pearly *Nautilus*, remains as living representative of an enormous but otherwise extinct cephalopod fauna. We may *suspect* that the circulation, respiration and hormone control of the Nautiloidea were like those of *Nautilus* but we have little assurance that this was so. The relationships between the modern and fossil shells are,

however, so close that we may confidently transpose the results which we obtain on the mechanisms of the cuttlebone and the shell of *Nautilus* to those which operated on cephalopod shells of the Palaeozoic and Mesozoic seas. These relationships are shown diagrammatically in

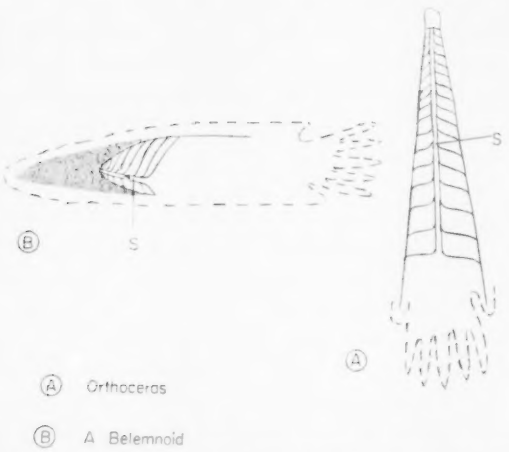

FIG. 15(a) Diagram of *Orthoceras*. The siphuncular tube, *S*, is shown passing through the chambers of the orthocone. The animal probably lived in the final chamber and may well have swum with the orthocone pointing upwards.
(b) Diagram of a Belemnoid. The calcareous mass at the posterior of the animal is stippled. The shell was probably internal. (After Hardy, 1956 and Morton, 1958.)

Figs. 15, 17, 18 and 19, where an early nautiloid's orthocone shell may be compared with those of *Nautilus*, *Spirula* and *Sepia* (the cuttlefish). In the orthocone a long thin shelly tube, the siphuncle(s), runs through the chambers. The siphuncle appears with but little change in *Nautilus* and *Spirula* and here we find that an artery and a vein run down its centre. It is clear that in *Nautilus* exchanges of substances between the insides of the chambers and the animal can only take place through the wall of the siphuncle. In these animals, including *Sepia*, parts of this wall appear likely from their mineral constitution to be permeable to liquids (Mutvei, private communication). Superficially, *Sepia* appears different from the others but again we have a chambered shell and, although it is true that the siphuncle is widely open and that the lower parts of the chambers of the shell have disappeared, a similar relationship between the living tissues and the shell persists. We may,

in particular, remark the close relationship between the epithelium lying against the siphuncular wall of *Nautilus* and that described as covering the siphuncular surface of the cuttlebone (Figs. 19 and 20).

We may now consider in turn what is known about the buoyancy of *Nautilus*, *Spirula* and *Sepia*.

2.2. *Nautilus*. Willey writes that one of Cuvier's regrets was not to have seen the inhabitant of the chambered shell of *Nautilus* which, from time immemorial, had been an ornament of the conchologist's cabinet. This regret is one most of us must share, for this beautiful living fossil is found only close to some of the larger islands in the Pacific. There it is eaten by the natives, but only when they can find

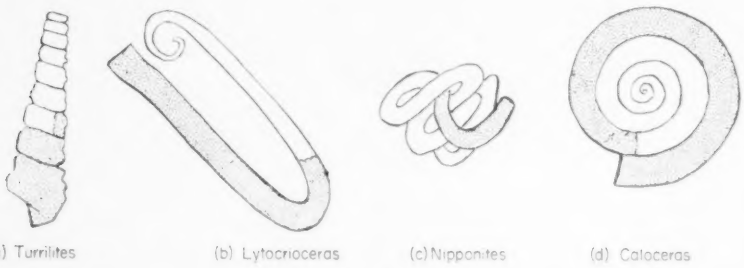

Fig. 16. Diagram of some coiled cephalopod shells in what may have been their natural position. The terminal chamber occupied by the body of the animal is shown stippled. (a) *Turrilites*; (b) *Lytocrioceras;* (c) *Nipponites;* (d) *Caloceras*. (After Trueman, 1940 and Morton, 1958.)

nothing better to eat. They, in their irreverence for antiquity, remind us of the Comoro Islanders who will cheerfully clean punctures in bicycle tyres with scales from the Coelocanth.

The nineteenth century saw studies on *Nautilus* by many of the foremost zoologists of Europe, yet in 1902 Willey could write that "so far as the finer anatomy and oecology of *Nautilus* is concerned it will be long before the Pieran spring is exhausted". The physiology, which will undoubtedly reveal some interesting curiosities, remains not so much unexhausted as unexplored; in particular a new study of the animal's buoyancy would certainly be repaid by discoveries of great interest.

About *Nautilus*, which is shown in Fig. 17, there is little new work to report but we may usefully collect some observations relevant to this problem from the older literature. Willey reports that the natives of New Britain commonly fish for *Nautilus* in depths of 50–130 m, and Dean (1901) that near the Phillipines it is generally taken from below 100 m down to a depth which the local fishermen estimate as 450–700 m.

FIG. 17. *Nautilus*.

(a) The living animal in approximately its swimming position. It uses its siphon for swimming.

(b) The empty shell—rather less than half of the shell has been cut away. The siphuncle is the tube passing through the air chambers. The animal lives in the last chamber. Magnification $\times \frac{2}{5}$.

(c) Part of the siphuncle. The hatched part (X) represents the region of the shell through which liquids probably move. The epithelium against this region is shown in Fig. 20. The stippled part (*L*) represents a living part of the animal—an artery and vein pass up the siphuncle. The part limited by a continuous black line is the pearly part (*P*) of the shell which is almost certainly impermeable to liquids. (After Willey, 1902 and Dean, 1901.)

There is some doubt about this latter figure because the deepest soundings recorded here are 200 m although this might simply represent the maximum sounding limit of the survey ship. Very occasionally *Nautilus* is seen close to the surface whilst some indication that its maximum depth range may be considerable comes from the capture of a specimen by *H.M.S. Challenger* when fishing at over 600 m. According to Dean (1901) the living *Nautilus* moves up and down with great facility and several authors have drawn the conclusion that the living animal in its shell is very close in density to sea water.

The gas in the air chambers has secured great attention from many workers and all believed it to occupy an important role in the functioning of the shell. Vrolik (1843) reported that it contained more nitrogen than does air, but his measurements, often quoted by later authors, were made on gas taken from specimens stored for some time in spirits of wine. Willey attributed to the siphuncle great power of secreting gas and observed that when the animal becomes moribund it rises to the surface, owing to the abundant production of gas in the interior of the body. This gas, he says, is most likely to come from the "regurgitation" of gas through the siphuncular venous sinuses. He noted that the finest ramifications of the veins especially those in the mantle flap were displayed (by air) "with a clearness which could hardly be attained and certainly not surpassed by artificial injection". This observation might suggest that the gas in the chambers was under pressure at depth and expanded and escaped by way of the siphuncle when the animal was brought to the surface, but, when Willey perforated the shell of a living animal under water, gas only bubbled gently out as water entered, an observation which suggests that the pressure of gas was close to atmospheric. We do not know, however, at what depth these different animals had been living immediately before these experiments were made.

Willey makes the curious statement that the chambers are not individually airtight because they are perforated by the siphuncle but that they are made airtight and watertight by the animal, which completely closes up the siphuncular entrance to the chambers. He does not seem to have considered that at depth great forces would be needed to control the water content of the chambers. This difficulty was well understood by Pruvot-Fol (1935) who suggested that gas produced by the siphuncle only partially pushed liquid from the chambers and that this gas was acted on directly by the external pressure, the gas spaces presumably decreasing in volume as the animal went deeper. She supposed that the buoyancy of the animal, although imperfectly regulated, was sufficient to aid swimming and compared the gas in the air chambers to gas trapped in an inverted glass.

One of the puzzles of the shell of *Nautilus* is set by the central position of the siphuncle in the air chambers (Fig. 17). If the siphuncle is the only region where liquids and gases can exchange, then, in the normal swimming position, it would seem to be difficult for *Nautilus* to empty the "air-filled" chambers completely no matter what mechism were used for expelling liquid. All the liquid might, of course, leave the newest-formed chamber across the septum separating it from the animal proper, the chamber being then sealed against further entry of liquid. This would presumably have to take place before the wall of the chamber became pearly and impermeable; an unlikely time because the septum would then be in a weak unfinished state, and ill-suited to stand any differences in pressure which would arise between the sea and the gas when the animal changed its depth. Willey and Pruvot-Fol both suggest that liquid is expelled from a chamber by the introduction of gas from the siphuncular wall of the chamber, but it now seems more likely that liquid is first removed through the siphuncular wall by some osmotic mechanism and that later gases diffuse into the space so created. This, as we shall see later, is what happens in the cuttlebone. On either hypothesis it is hard to see how a chamber with a centrally placed siphuncule could be completely emptied. Water vapour might of course condense on the siphuncular wall and then be extracted from the chamber osmotically; the siphuncular wall facing into the chamber would then have to be kept very dry.

Perhaps like the cuttlefish, *Nautilus* can control the amount of liquid in its shell and this may even act as a ballast helping to determine the animal's posture. If it were true that the chambers could contain some water then many of the puzzles of the buoyancy of the fossil cephalopods could be readily explained, although it would then become rather difficult to make exact calculations of the buoyancy of the shell in any particular animal.

2.3. *Spirula*. This cephalopod, shown in Fig. 18, is found principally in the warm subtropical and tropical oceans, being most common above the steep declines from the continental shelf to the abyssal region. It was for long known only from the shells of dead animals, which can drift enormous distances and are cast ashore in almost all parts of the world.

In 1845 Gray could properly entitle a paper in the *Annals and Magazine of Natural History*, "On the animal of *Spirula*", and until the *Dana* expeditions in the 1920's only a few specimens had been captured. Our knowledge of the biology and distribution of *Spirula* rests almost entirely on observations made during, and the analysis of catch made after, the *Dana*'s very fruitful voyages (Schmidt, 1922; Bruun, 1943). Because of its close relationship with the numerous fossil

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ammonoids the few *Spirula* caught in the last century were most carefully studied and Bruun (1943) could note that more was known about the anatomy and morphology of *Spirula* than of very much more common cephalopods.

The small gas-filled shell is clearly an organ of buoyancy which brings the animal fairly close in density to sea water. Gray (1845) writes "I am informed by M. Clausen that he had several specimens alive and kept them for some time in two vessels filled with sea water and that they had the power of ascending and descending at pleasure"; and Schmidt (1922) noted that "live *Spirula* when resting rose to the surface, but they are not so overcompensated by air they could not move down freely". The shell lies posteriorly and the animal generally hangs vertically with its head and mouth pointing downwards (Fig. 18). The animal can float gently or use its siphon and stern fins to move jerkily in any direction. At the posterior end is a small bead-like organ which emits a pale yellowish green light which has been seen to shine continuously for hours and perhaps serves to keep the animals together in schools.

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The principal problem with respect to buoyancy is how the gas spaces are produced and maintained. Of first importance will be the depth range over which the animal can live, for this will determine against what pressures the mechanism responsible for the production and maintenance of the gas spaces will have to work. The *Dana* caught 193 individual specimens of *Spirula*, all in mid water and, from an analysis of the catches made by nets at different depths Bruun concludes that although in certain places it was caught at around 100 m it normally lives below 200 m. The lower limit at which it was caught was about 1750 m, the young specimens with a mantle length $\leqq$ 5 mm were concentrated between 1000 and 1750 m. The adults may perhaps visit the bottom to lay eggs. Thus we may account for specimens caught in dredges by the *Challenger* and the *Blake*. From the former, Huxley and Pelseneer, quoted by Murray (1895), had concluded that *Spirula* swam close to the bottom and, from the latter, Agassiz (1888) that it lived with its posterior extremity buried in the mud.

Bruun discusses at length the hypothesis that, somewhat like the fish's swimbladder, the pressure of the gas within the shell matches the external hydrostatic pressure at the depth where the animal lives. He advances several arguments which would make this a reasonable assumption. He notes that the septa separating the chambers are bent in a sphere inwards and are thus well fitted to stand pressures—especially from the inside. He calculates that the expenditure of energy required to produce the gas pressures needed would not be very large. Finally he tested shells, from dead animals, under pressure and found

that they generally burst under pressures of between 25 and 40 atm although some stood pressure higher than 65 atm but cracked at 80 atm. This would imply that if the gas within the shells were at low pressure most of these shells would have burst at 400 m whilst all would

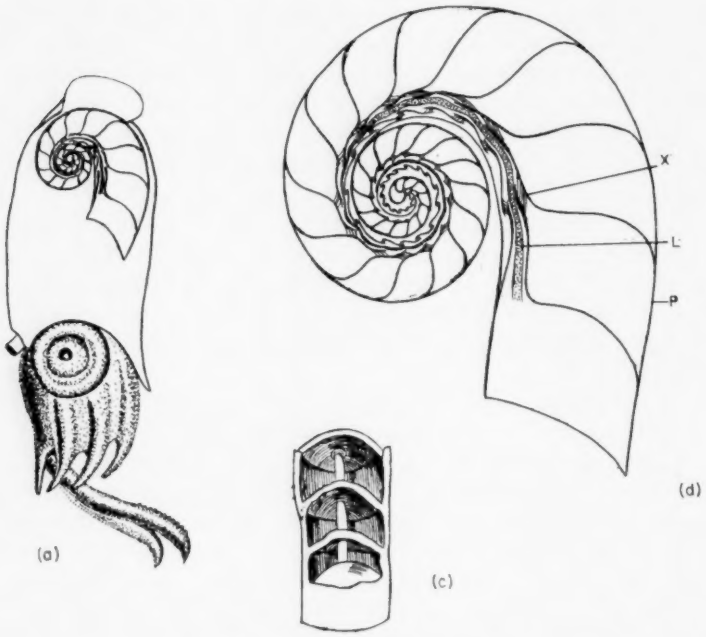

FIG. 18. *Spirula spirula*. Diagrams.

(a) The animal in its natural swimming position. The position of the shell which is internal is shown, as are the two small stem fins. Approx. natural size.

(b) The shell. The hatched part (X) represents the region through which liquids probably move. The stippled part (*L*) is the siphuncular tube with its artery and vein. The continuous lines (*P*) the pearly part of the shell which is probably impermeable to liquids.

(c) Some chambers opened to show the position of the siphuncle. (After Chun and Brauer, 1914, Bruun, 1943, Cooke, Shipley and Reed, 1895 and Mutvei private communication.)

have burst at about 750 m, both of which depths are much less than the 1750 m at which *Spirula* is sometimes caught. A high pressure of gas inside the shell could, of course, prevent such bursting, and Bruun says that "if a newly hatched *Spirula* filled its shell with air at a depth of, e.g., 1000 m it will not suffer from making vertical movements of several

hundreds of metres up or down". These arguments for a high internal gas pressure are not conclusive for the shells in the living animals may well be stronger than those studied and may perhaps stand up to greater pressures, and arguing against this hypothesis are the facts that shells from dead animals are never found broken by gas explosion and live specimens never have a burst shell after being hauled in from the deep. This, Bruun says, may be explained by the gas escaping through fine pores in the shell or siphuncle. Alternatively, Bruun (reported by Lane, 1957) has suggested that the surplus air may be carried away by the blood of the animal.

He notes, however, that "the most simple explanation could be given if it could be proved that the pressure inside the shell is always at about 1 atm . . . that a chamber when finished is first filled with fluid which is absorbed and replaced by air by a fairly simple osmotic mechanism". The greatest difficulty in this latter hypothesis would seem to be that of providing simple osmotic forces of up to 175 atm, since even if all salts were pumped out of the fluid within the shell the simple osmotic force is unlikely to exceed that between sea water and distilled water, which is about 24 atm. The water might, of course, be pumped out at lower depths and the chambers then sealed. Telling against this is the observation that the younger animals live predominantly in the deeper zone at 1000–1750 m. Whatever mechanism is used the water-permeable parts of siphuncle seem to be very inconveniently placed for the extraction of water from the newest chambers at least when the animal is in its usual swimming position.

2.4. *Sepia officinalis.* Shortly after the opening at Arcachon of the first important laboratory in Europe to be devoted to marine research, Paul Bert undertook a general study of the physiology of the cuttlefish *Sepia officinalis*. He promised himself that he would not abandon this task until he knew as much about *Sepia* as was then known about the physiology of the frog (Bert, 1867). He chose *Sepia* because it is more plentiful than the octopus and tougher than the squid. In Bert's memoir the cuttlebone is quickly noticed. He writes "Les seiches pénètrent dans le bassin d'Arcachon vers la fin du mois de mars. Elles y arrivent en grandes troupes, que suivent nombre de cétacés delphiniens, avides d'une pareille proie. Ceux-ci leur tranchent fort habilement la tête, au niveau de l'os dont leur dents redoutent probablement le contact. Aussi au mois de mars et d'avril voit-on arriver à la côte une grand quantité de seiches décapitées." Of experiments on the cuttlebone he attempted little but he did make one experiment of importance. He collected small bubbles of gas from within the cuttlebone by gently grinding it under water and showed that this gas was principally nitrogen containing only 2–3 per cent of oxygen. He believed it probable

that the composition of the gas would vary with circumstances in a way analogous with that which Moreau had shown for the gas in the swim-bladder of a fish.

The standard description of the dead cuttlebone was made 20 years later by Appellöf (1893) and it is from his work and our own observation that the following account is drawn. In a large adult the cuttlebone consists of about one hundred lamellae of calcified chitin placed one above the other and held about 0·6 mm apart by numerous irregular vertical pillars. The spaces between the lamellae form chambers which are themselves divided by about six thin membranes running parallel to the lamellae. These chambers are sealed laterally and anteriorly by the thick calcified layer forming the curved dorsal surface of the cuttlebone. They are successively laid down, one below the next, as shown in Fig. 19 and Plate II. Here the surface *AB* of Fig. 19b represents the posterior margins of the turned-over ends of the lamellae. This surface, which has a rich circulation, and which assumes a special importance in later studies on physiology, represents the effective part of the siphuncle in *Sepia*. Since Bert, the physiology of the cuttlebone has received little attention and the following account is largely based on the recent work of Denton and Gilpin-Brown (1959 and 1960).

The cuttlebone's volume is about 9 per cent of the cuttlefish's total volume, its density is around 0·6 so that in a 1000 g animal the cuttlebone will give an upthrust of 40 g which will just about balance the excess weight in sea water of the rest of the animal. The first clue that the cuttlebone was anything but a dead unchanging organ came from a study of animals which had been kept in aquaria for some time. It is well known that some cuttlefish sometimes seem to be very buoyant and find difficulty in staying at the bottom of a tank. Two groups of animals were taken, one in which the animals appeared to be very buoyant and another in which the cuttlefish could rest on the bottom of the tank without effort. When anaesthetized the former were found to be less dense than sea water, the latter denser. No bubbles of gas were found either in the mantle cavity or in the softer parts of the body and the bodies without cuttlebones were all of about the same density. The cuttlebones of the two groups on the other hand differed markedly in density. The cuttlebones from the "light" animals having densities close to 0·5, those from the "heavy" animals around 0·65. There was no difference between the two groups in the weight of dry matter per unit volume of bone, this remained always close to 38 per cent. The differences in density of the cuttlebones were entirely attributable to differences in the percentage of liquid which they contained; thus, a cuttlebone of density 0·7 contained about 30 per cent of liquid whereas a cuttlebone of density 0·5 contained about 10 per cent liquid. As the

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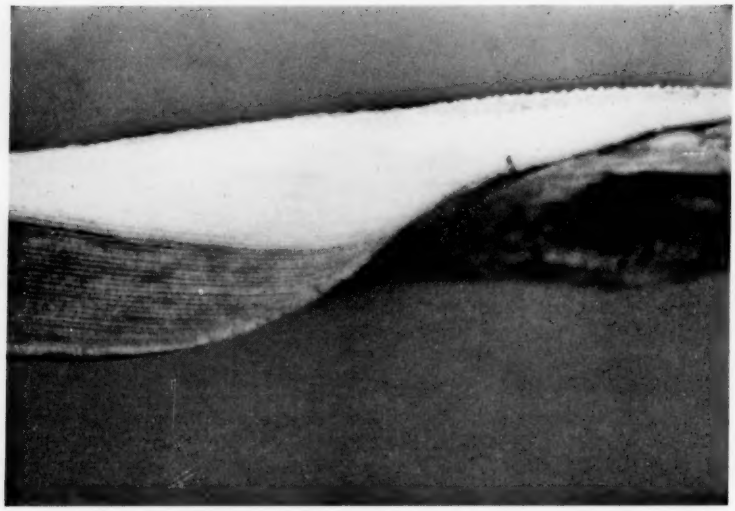
PLATE I. Radiographs comparing the degree of ossification of *A*, *Chauliodus sloanei*, *B*, *Gadus minutus*, *C*, *Gonostoma elongatum*, and *D*, *Gadus minutus*. Of the two specimens of *G. minutus* the second, *D*, is much smaller than either of the bathypelagic fish. The swimbladders of *G. minutus* appear as light areas in the body. Magnification × $\frac{2}{3}$. (After Denton and Marshall, 1958.)

[*facing p. 222*



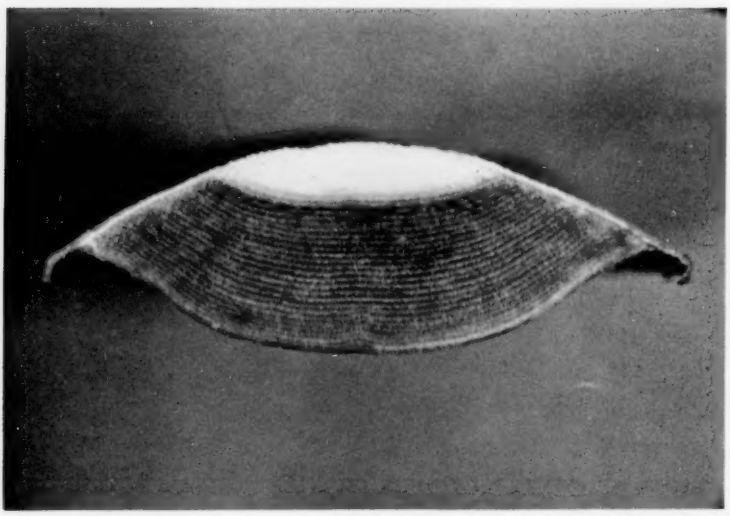


PLATE II. *Sepia officinalis*. *A*. Longitudinal section of cuttlebone (shown *AB* of Fig. 18a) Magnification $\times \frac{2}{3}$. *B*. Transverse section of cuttlebone (shown *CD* on Fig. 18a). A number of lamellae have been punctured from the ventral side. Gas has been removed through the hole by vacuum with the bone under sea-water containing ink. Atmospheric pressure was finally restored and inky water has filled the chambers from which gas has been removed. (After Denton and Gilpin-Brown, 1959 and 1961).

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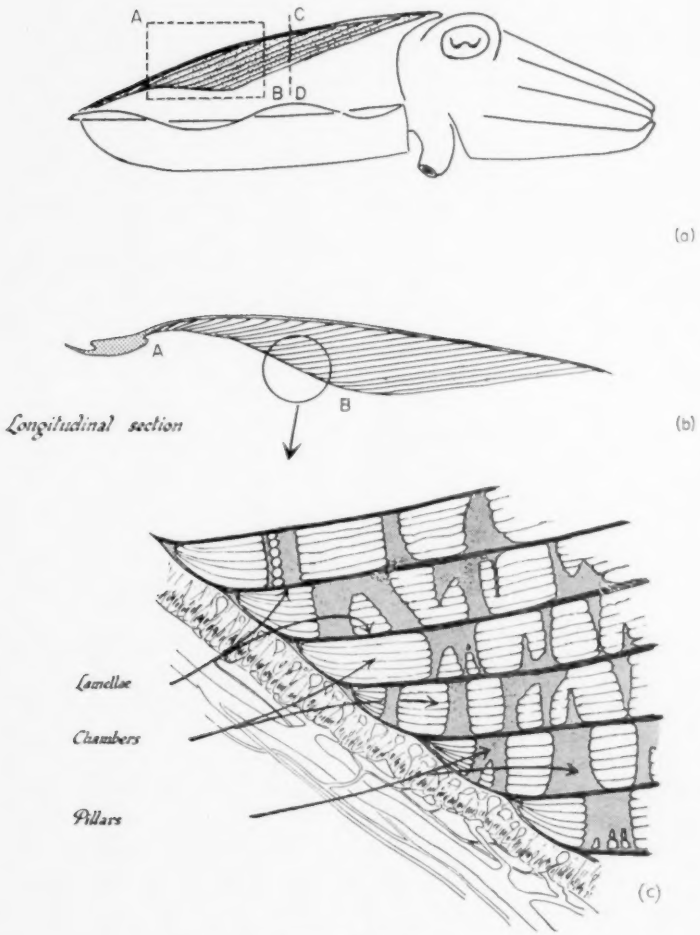

FIG. 19. *Sepia officinalis*. Diagrams.

(a) The animal in a natural swimming position, the cuttlebone is internal but a longitudinal section of the cuttlebone is shown to indicate its position in the animal. Magnification $\times\frac{1}{3}$.

(b) A longitudinal section of the cuttlebone. The siphuncular surface *AB* is probably that through which liquids move.

(c) Section of cuttlebone showing the epithelium lying on the surface *AB* of (b). In this epithelium the ampullae close to the bone which drain to the veins can be seen. (After Denton and Gilpin-Brown, 1959 and 1961.) The areas *AB* and the line *CD* of (a) indicate sections shown on Plate II.

percentage of liquid decreased the volume of the gas-filled spaces increased and the bone became less dense.

It was later shown that appreciable changes in buoyancy of *Sepia* can take place in relatively short times.

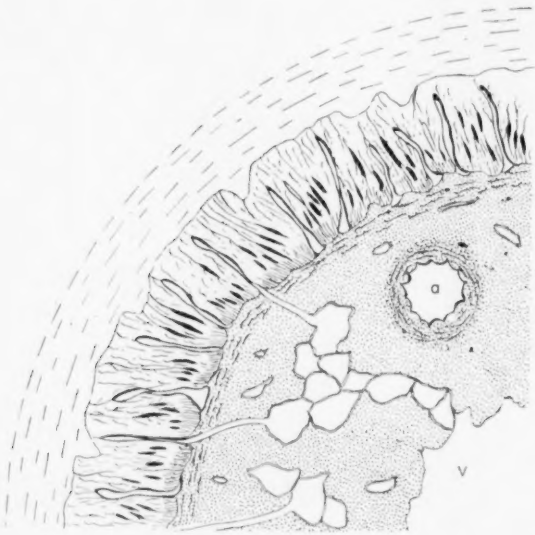

FIG. 20. *Nautilus*. Diagram of the cross-section of the siphuncle: a, artery; v, vein. The epithelium against the siphuncular wall is very like that found against the siphuncular surface in *Sepia* (Fig. 19). (After Willey, 1902.)

The behaviour of well-fed cuttlefish is strikingly affected by light. When the light is bright they usually bury themselves in the gravel at the bottom of their tanks whilst after twilight they come out of the gravel and swim around until dawn (Fig. 21). A cuttlefish can be shepherded into a box without being much disturbed and changes in density of such animals were studied by weighing them *under sea-water* in a watertight box which was filled with sea water. Now if, for example, an animal weighs minus 3 g in sea water then the weight of box plus animal plus sea-water will be 3 g less than the weight of box filled with sea-water only. Animals kept in artificial light and dark showed quick changes in density which could amount to over 1 per cent (Fig. 22). These could scarcely come from changes in protein content for they would imply oscillations in total protein of about 25 per cent and this in animals weighing $\frac{1}{3}$ kg and eating about two prawns per day each. Such changes could only come from a change in the volume of the gas

space which the cuttlefish contained. There was no trace of gas within the mantle cavities and when the experiment was stopped the densities of the cuttlebones were found to be those predicted on the hypothesis that the cuttlebone was the site of the density change. Thus, a high density of cuttlebone was found for an animal which had been in the light, and a low density for an animal which had been in the dark.

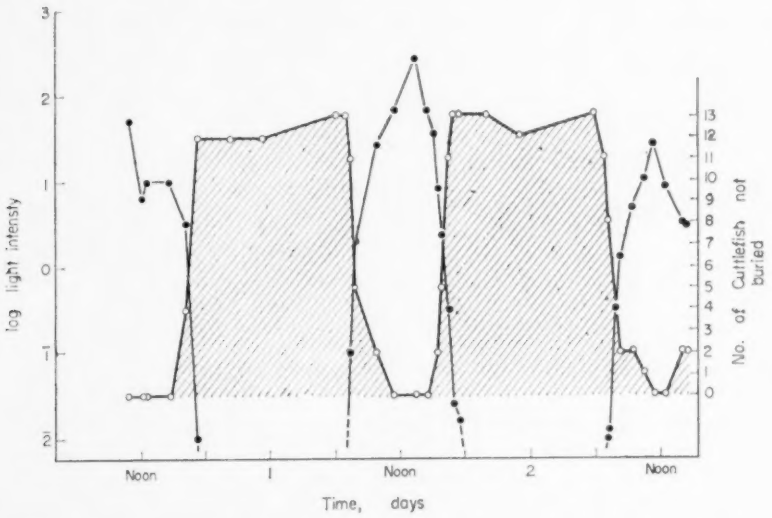

FIG. 21. *Sepia officinalis*. Diurnal changes in the behaviour of the animals. ○ Number of animals *not* buried, (Total number of animals 13) • Changes in natural light intensity on a logarithmic scale. The light intensity was not far from that to be found in the sea at 20 fathoms but its spectral composition would be different. The cuttlefish can be seen to bury itself in the day-time and come out when the light is dim. (After Denton and Gilpin-Brown, 1960 and 1961.)

These experiments show that the cuttlefish can speedily change its density by pumping water in and out of the cuttlebone. In the experiment just described the period of darkness lasted longer and was more complete than in nature, and the increase in buoyancy in the dark was doubtless thereby exaggerated. There can be little doubt, however, that in the sea similar though smaller diurnal cycles of buoyancy take place and help the cuttlefish to lie at the bottom in the day and hunt at night.

The cuttlebone's dorsal surface is covered with a thick layer of calcified chitin, and the anterior ventral surface runs parallel to the

many layers of calcified chitin of the lamellae. Anatomically, therefore, the most likely place for liquid exchanges between the cuttlebone and the rest of the animal would be the region *AB* of Fig. 19b (see also Plate II) formed of the turned-over ends of the lamellae. This region of the siphuncle, which is the only possible surface for the exchange of substances between gas-filled chambers and animal in *Nautilus* might be expected to be the site of the known exchanges in *Sepia*. The yellowish-coloured membrane covering the region *AB* has, moreover, not only a copious blood supply but also contains numerous ampullae

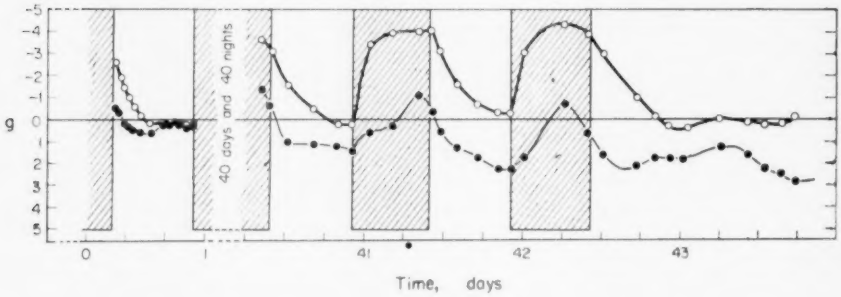

FIG. 22. *Sepia officinalis*. Changes of weight in sea water of two specimens. The upper curve is for an animal weighing around 330 g, the lower curve for an animal weighing around 260 g. The ordinate shows the weight of an animal in sea water—negative weights mean that the animal was less dense than sea water. The dark areas indicate the times of darkness. (After Denton and Gilpin-Brown, 1960 and 1961.)

close to the bone which are connected by small ducts to the veins (Fig. 19c). In histological sections the ampullae and the ducts leading to them contain no blood although blood can be seen in very much smaller blood vessels. There is, therefore, a special drainage system in the very region where we suspect exchanges of liquid to take place in the living animal. Such reasonings based on anatomy are confirmed by experiment. If a newly dissected cuttlebone is placed under reduced pressure a watery liquid flows from the surface *AB* (Fig. 19b) but from nowhere else.

Experiments such as these show that the cuttlebone is not an impermeable structure and, we may well ask how liquid can be held out of the cuttlebone in life. The cuttlefish is not a very deep-living animal—off Plymouth it is most frequently taken between 30–80 m and it is thought to go down occasionally to about 200 m. It may then be commonly exposed to pressures of around 8 atm and occasionally to

pressures of 20 atm. These pressures will tend to push water into the cuttlebone, and any mechanism which we suggest must be capable of balancing them.

Before going further we ought to know the relationship between one chamber in the cuttlebone and the others. To study this the lower chambers of the cuttlebone were punctured from the ventral surface and the cuttlebone placed under sea water containing ink and then exposed to a vacuum. When no more gas came from the hole atmospheric pressure was restored and the inky sea water allowed to fill the space from which the gas had been taken. The results of such an experiment are shown in Plate II. It will be seen that the vacuum only extracted gas from the punctured chambers but that these chambers filled up completely with ink when the atmospheric pressure was restored. The chambers are then independent of one another yet within one chamber gases and liquids are free to move.

Returning to the problem of finding a force to balance the external hydrostatic pressure let us first examine Bert's suggestion that the cuttlebone is similar to the swimbladder and see whether at depth it contains gas under high pressure. When cuttlebones quickly dissected from cuttlefish newly hauled aboard ship from about 80 m depth were placed under water and punctured with a needle no stream of bubbles emerged from the hole. We should have expected this to happen if the cuttlebone contained gas under pressure. Instead of bubbles of gas coming from the hole, water rapidly entered the cuttlebone, showing that the pressure of gas within the cuttlebone was not more, but less, than atmospheric. This experiment might be explained by the rather unlikely assumption that there exists a gas in the cuttlebone which is very soluble in sea-water. This assumption was shown to be false by puncturing a cuttlebone in air in a closed system when, instead of water, an equivalent volume of air entered the bone. The degree of partial vacuum inside the cuttlebone may be estimated by weighing a cuttlebone in sea-water both before and after puncturing all the chambers under sea-water (Fig. 23). A shell in which the partial pressure is lower will take up proportionately more liquid on puncturing.

A group of cuttlefish were freshly caught and studied aboard *R.V. Sarsia*. These cuttlefish had cuttlebones whose densities fell within the narrow range 0·57–0·64 (mean 0·62, s.d. = 0·02, $N = 17$). A second group of animals which had been kept in an aquarium for a week or two exhibited the much wider range of cuttlebone densities 0·48–0·71 (mean = 0·60, s.d. = 0·06, $N = 35$). Clearly some of the cuttlefish had become less dense and some denser since capture. Fig. 23 shows the change in density of a number of cuttlebones of the second group on puncturing. Here we see that no matter what its density before

puncturing, the cuttlebone takes up an amount of water which will bring its final density close of 0·73. This indicates that when a cuttlebone becomes less dense the pressure of the gas within it falls, and that

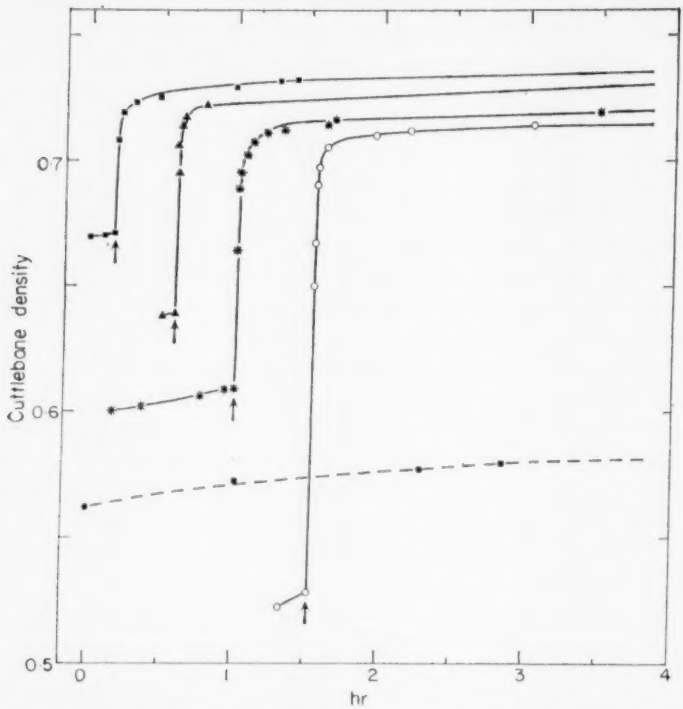

FIG. 23. *Sepia officinalis*. Freshly dissected cuttlebones of various densities were punctured under sea water. These became denser as water flowed into the chambers to fill the partial vacuum which had existed there. It can be seen that no matter what the starting density the final density was always close to 0·73. The mass of gas within the cuttlebone remains therefore approximately constant when liquid is pumped in or out of the cuttlebone. The times of puncture are shown by the arrows. (After Denton and Gilpin-Brown, 1959 and 1961).

when a cuttlebone becomes more dense the pressure of gas rises, and that within the duration of these experiments, the mass of gas per unit volume of bone remained almost constant whatever the density of cuttlebone. The pressure of gas which varies about 0·8 atm never becomes greater than atmospheric and since when water is expelled the gas pressure falls, there can be no question of an evolution of gas raising the pressure and expelling liquid.

Some other mechanism than gas pressure must therefore be sought,

both for creating and maintaining the gas space and for moving water into and out of the cuttlebone.

Liquid can be taken from cuttlebones under liquid paraffin by reducing the external pressure. Such liquid, taken aboard ship, was found by measurements of freezing points to be hypotonic to sea water and to be principally a solution of sodium chloride. This suggests that simple osmotic forces between this liquid and the blood (the latter is almost isotonic with sea water, Robertson (1949, 1953)), play a role in holding liquid out of the cuttlebone when the animal is at depth. In animals taken where the depth of water was 70 m this osmotic difference could balance about 5 atm of the extra 7 atm pressure at the sea bottom. Thus, simple osmotic forces account for most of the forces balancing the external hydrostatic pressures. The gas inside the cuttlebone is probably simply that which diffuses from the tissues into spaces made by some osmotic mechanism. The pressure of nitrogen, about 0·8 atm, approximately matches that dissolved in sea water. The oxygen which might diffuse into the gas space is perhaps used metabolically.

A simple (or an anomalous)* osmotic force may stop fluid diffusing into the cuttlebone but it cannot balance the crushing effect of the hydrostatic pressure of the sea. The cuttlebone with its lamellae held apart by numerous pillars is, however, well designed to sustain compression. It is very strong and one, dissected from the animal and tested in a pressure tank, only imploded at a pressure corresponding to a depth of 250 m; a depth appreciably greater than that at which the animal lives.

2.5. *Summary.* The study of the buoyancy of the chambered shells of cephalopods is in an interesting state. For *Sepia* it is clear that liquid is moved in and out of the cuttlebone by some kind of osmotic force which works against the hydrostatic pressure. It has not been demonstrated for *Sepia* that *simple* osmosis is the only force used to control the diffusion of water in and out of the cuttlebone although it seems to play an important role. The gas within the cuttlebone appears to play a passive role in the mechanism of the cuttlebone; it probably merely diffuses into a space created by the extraction of liquid. The shell is strong enough to stand the external hydrostatic pressure which in *Sepia* may perhaps reach 20 atm.

Anatomically and histologically the shells of *Nautilus* and *Spirula* are so closely related to *Sepia* that it seems likely that they all work in the same way. *Spirula* is found at depths down to 1750 m and here simple osmosis could not possibly be the only force holding liquid out of the shell.

* Bayliss (1959) gives examples of the magnitude of force which might arise by anomalous osmosis.

Because of its availability experiments on *Sepia* hold the best hope of finding the mechanism controlling the buoyancy of the chambered cephalopod shell. Any mechanism suggested ought to be one which could work in *Nautilus* and *Spirula*, both of which have a much greater depth range.

It seems possible that the fossil cephalopods resembled the modern *Sepia* in having chambered shells containing liquid as well as gas and the common assumption that the chambered shells of these fossil animals contained only gas (this is often assumed in calculation about their posture and buoyancy) is perhaps invalid. Fortunately, the pearly *Nautilus* still affords us a last chance of studying this problem.

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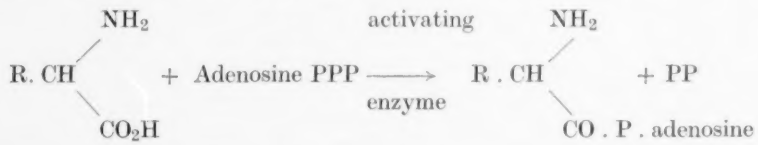
CYTOPLASMIC PARTICLES AND THEIR ROLE IN PROTEIN SYNTHESIS

1. Introduction

An informal discussion on this subject was held on 28–29 March 1960 in the University of Reading, under the auspices of the Colloid and Biophysics Committee of the Faraday Society. A number of short papers were read and discussed, and the abstracts written by the authors give an excellent survey of recent work in this field, which it is thought will be of interest to readers of *Progress in Biophysics and Biophysical Chemistry*. The scope of the papers presented at this meeting was in fact somewhat more extensive than is implied by the title, in order to provide links with other aspects of the mechanism of protein synthesis.

Much of the background of this discussion will be found in the article of R. B. Loftfield (1957) in Vol. 8 of the series. Evidence was quoted there for the now widely held view that the microsomes of some mammalian cells consist of a series of membranes containing phospho-lipids and lipo-protein, which are often covered with dense ribonucleoprotein particles (ribosomes) about 140 Å in diameter. In some cells (e.g. many bacteria) the particles appear unattached to any membranes. Such membranes are by no means seen in all cell preparations, however, but appear to be more frequently observed in secretory than in non-secretory cells. A number of investigations of the size and structure of these particles, particularly those derived from bacteria, were described. In certain cases it has been found that the larger particles are aggregates of smaller ones, and the state of aggregation depends on the concentration of magnesium ions and on the other circumstances (Petermann; Huxley and Zubay). However, Hanzon and Sjöstrand (1959) have suggested that the compact particles observed under the electron microscope after the usual staining procedures with osmium tetroxide may be artifacts, since the particles are not seen after a procedure in which whole cells, normally found to contain microsomal particles when fixed with buffered osmium tetroxide, are frozen dried before examination.

With regard to the function of these particles, it will be recalled that it is now generally recognized that the first stage of protein synthesis is the activation of the amino acids by ATP. This may be represented:



Hoagland, Keller and Zamecnik (1956) have given evidence for the view that the activated adenyloamino acid is then attached to the RNA of the

supernatant fraction (S-RNA) from which it is transferred to the microsome in which the actual synthesis of the protein occurs.

However, although the steps of this process are supported by experiments, it has been shown that other possible processes occur, e.g. in the presence of ATP and various energy providing systems and amino acids the latter may become directly incorporated into the microsome without the apparent intervention of substances present in the supernatant (RENDI and HULTIN, 1960). This can also occur when much of the phospholipid and part of the protein of the microsome has been removed by various agents (see COHN and BUTLER, ZALTA, KORNER below). It has also been demonstrated that isolated mitochondria can carry out the complete synthesis of cytochrome-c (SIMPSON, ROODYN).

Other possible mechanisms arise from the observation of HENDLER (1958, 1959) that lipid–amino acids complexes occur in a variety of tissues; this is supported by the preparation by HUNTER (below) of such complexes from *B. megaterium*.

J.A.V.B.

The following abbreviations are used: RNA, ribonucleic acid; RNP, ribonucleoprotein; S-RNA, supernatant RNA; ATP, adenosine triphosphate; CTP, GTP, UTP, cytidine, guanosine, uridine triphosphates; ADP, CDP, GDP, UDP, similar diphosphates; AMP etc., similar monophosphates; ER, endoplastic reticulum; DMNA, dimethylnitrosamine; DNA, deoxyribonucleic acid; AA, amino acid.

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II. THE RESOLVING POWER OF CELL FRACTIONATION TECHNIQUES

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THE development of techniques for the fractionation of intracellular constituents has opened up a new field of investigation, the possibilities of which have so far been exploited very incompletely. One important factor of progress is to increase the resolving power of the methods. In this respect, the classical scheme of division of homogenates into four fractions is generally inadequate for unequivocal localization of enzymes.

One approach is to increase the number of fractions, keeping the principle of separating the fractions on the basis of differences in sedimentation velocity. This may be done either by isolating washed sediments as in the original method, or by density gradient centrifuging. Density equilibration in a gradient provides another approach, which permits separation on the basis of differences in density. Both approaches offer a number of variants, since the sedimentation velocity and density of particles may be influenced selectively by changes in the composition of the medium.

Several of these new techniques have been used in this laboratory and applied to the localization of some thirty different enzymes in rat liver. Several new groups of cytoplasmic particles, each corresponding to a small

fraction of the total tissue nitrogen, have already been identified in addition to the true mitochondria and to the endoplasmic membranes and their attached ribosomes, which form the main components of the classical mitochondrial and microsomal fractions respectively.

(A review covering the above subject has recently been published under the title "La localization des enzymes dans les éléments figurés des cellules" (DE DUVE, 1960).)

III. STUDIES ON SOLUBLE AND MICROSOMAL RNA IN CONNECTION WITH PROTEIN SYNTHESIS

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SOLUBLE polynucleotides, isolated by phenol extraction from rat liver cytoplasm, have been fractionated on the weak anion exchanger Ecteola. One of the four S-RNA fractions only seemed to be involved in the following two metabolic transfer reactions:

(*a*) Amino acid transfer to the microsomes

(*b*) Transfer of label from ^{32}P-S-RNA to microsomal RNA.

The efficiency of the latter reaction was about 17 per cent based on the isotope content of the added polynucleotides.

Ecteola fractionation of microsomal RNA, isolated in a similar fashion from deoxycholate-insoluble particles yielded three fractions, one of which acted as acceptor of label from ^{32}P-S-RNA during the *in vitro* incubation of microsomes with soluble polynucleotides. This selective metabolic transfer resulted in the attachment to microsomal RNA by bonds which were not disrupted by the combined treatment of microsomal RNA with 8 M urea and EDTA. A search for possible intermediates of smaller size has been made but without success so far.

It may be concluded, however, that the intermediate formation of smaller poly-, oligo- or mononucleotides, if actually involved in the transfer reaction, does not seem to depend on the presence of enzymes residing in the soluble part of the cell.

IV. SOME DATA ON THE OCCURRENCE AND COMPOSITION OF NUCLEOTIDE-BOUND CARBOXYL-ACTIVATED PEPTIDES: THEIR POSSIBLE RELATION TO THE BIOSYNTHESIS OF PROTEINS

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THE occurrence of dialysable carboxyl-activated peptide-nucleotide compounds in extracts of bakers' yeast was reported by a group of investigators from our laboratory about three years ago (KONINGSBERGER *et al.*, 1957a, 1957b). Our study of the occurrence of these compounds indicated that they can be isolated from both the soluble and the particulate fractions of the cell (KONINGSBERGER *et al.*, 1957b; GRINTEN *et al.*, 1958; KONINGSBERGER, 1959;

WEIL *et al.*, 1958). Recently, the isolation of similar compounds from various tissues and micro-organisms synthesizing proteins was reported by a number of authors. Considering the data on amino acid activation (DAVIE *et al.*, 1956) and on the transfer of activated amino acids to ribonucleic acid (S-RNA) from the soluble fraction of the cell (HOAGLAND *et al.*, 1957, 1959), our attention was drawn to the possible occurrence of a "pre-microsomal" condensation of these activated amino acids to small, carboxyl-activated peptides, which could occur on the S-RNA. An indication of such a condensation may be seen in the occurrence of carboxyl-activated peptides in preparations of S-RNA from both bakers' and brewers' yeast (KLOET *et al.* 1960). The effect of such peptides on the induced α-glucosidase synthesis by protoplasts of brewers' yeast is being studied: preliminary experiments seem to indicate that some peptides have a stimulating and others an inhibitory effect on this synthesis.

V. NUCLEOSIDE-TRIPHOSPHATE DEPENDENT SYNTHESIS OF PEPTIDES BY THE PURIFIED "AMINO ACID INCORPORATION ENZYME"

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A SOLUBLE enzyme preparation, purified from *Alcaligenes faecalis*, promotes the incorporation of amino acids into peptide bonds when added to cell membrane fragments obtained from the same bacteria (BELJANSKI and OCHOA, 1958; BELJANSKI, 1960a). This purified enzymatic preparation which we have called the "amino acid incorporation enzyme" does not exhibit myokinase, pyrophosphatase, polynucleotide phosphorylase or amino acid "activating enzyme" activities.

(1) *Exchange between nucleotides*

The "amino acid incorporation enzyme" catalyzes a Mg^{++} dependent transfer of the terminal phosphate from each of the four nucleoside-triphosphates to the homologous nucleoside diphosphate (ATP-ADP; GTP-GDP; UTP-UDP; CTP-CDP). Thermal inactivation experiments with this enzyme have given data suggesting that this system contains four different and specific activities each capable of catalyzing the transfer of phosphate between one pair of homologous nucleotides. There is no transfer of phosphate between heterologous nucleotides (BELJANSKI, 1959, 1960a). All four exchange activities appear to be necessary for amino acid incorporation in the complete system, because when one of the exchange activities is missing in the purified "amino acid incorporation enzyme" no stimulation of amino acid incorporation is observed.

(2) *Liberation of phosphate*

When the purified "amino acid incorporation enzyme" is incubated with each of the four nucleoside-triphosphates no liberation of phosphate occurs.

When, however, amino acids are added to the incubation mixture, liberation of phosphate takes place. There is a high degree of amino acid specificity in the liberation of the terminal phosphate from nucleoside-triphosphates. For example, in the presence of valine, phosphate is liberated from CTP, but not from ATP, GTP, or UTP. Leucine allows liberation of phosphate from CTP and UTP, but not from the others. Chloramphenicol prevents the liberation of orthophosphate.

(3) *Peptide formation*

The amino acid dependent liberation of the terminal phosphate from nucleoside-triphosphates suggested the possibility of the formation of peptide bonds by the purified "amino acid incorporation enzyme" alone. This enzyme has been incubated with a mixture of 18 L-amino acids, several of which were labelled with ^{14}C, in the presence of one nucleoside-triphosphate (UTP, CTP, GTP or ATP). After stopping the reaction by heating at 100° for one minute, paper chromatography and autoradiography of the supernatant showed the presence of several new radioactive spots with Rf values different from that for free amino acids. The newly formed material representing about 20 per cent of the total radioactivity has been identified as peptides by chemical and enzymatic means (BELJANSKI, 1960b).

VI. ELECTRON-MICROSCOPE STUDIES OF MICROSOMAL PARTICLES FROM *E. COLI*.

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MICROSOMAL particles were first observed in the electron-microscope by PALADE (1955) as a characteristic component of the cytoplasm of a number of mammalian and avian cells, and PALADE and SIEKEVITZ (1956a, b) and SIEKEVITZ and PALADE 1958a, b, c, 1959) have described a number of structural and biochemical studies on them, showing that their presence was associated with extensive protein synthesis. Such particles appear in sectional material as dense bodies having a diameter of about 150 Å; they are often attached to membranes of the endoplasmic reticulum, but are also to be found free in the cytoplasm.

Rapidly growing bacterial cells are also sites of large scale protein synthesis, and they too contain large numbers of microsomal particles in their cytoplasm, most usually not attached to membranes. The extraction and characterization of such particles from the bacteria *E. coli.* has been described by TISSIERES and WATSON (1958) and by TISSIERES *et al.* (1959). These authors have shown that the microsomal particles from *E. coli.* can exist in a number of different stable forms, depending foremost on the concentration of free magnesium ions, and characterized by sedimentation constants of 100 S, 70 S, 50 S, and 30 S. These different forms, identified in each case by sedimentation analysis, have been investigated here by electron

microscopy, using both negative and positive staining. Using negative staining we found that the 100 S particles consisted of two 70 S particles joined together as a dimer. The 70 S particles could be seen to contain two sub-units of unequal size with a well-marked cleft between them, and in the 100 S particles the smaller sub-units of the two 70 S particles were apposed. 50 S and 30 S particles were found to resemble closely the larger and the smaller sub-units of the 70 S particles. In addition, it was found that purified 50 S particles, when transferred to a medium in which 100 S particles were stable, formed dimers with a sedimentation constant of about 81 S.

The particles were not found to have a regular polyhedral form, in contrast to some small spherical viruses. The 50 S particles appeared to be very approximately dome-shaped. The 30 S particles had a flatter form with a rather irregular outline. In the 70 S particles, the smaller sub-unit was seen to fit like an overlapping cap on the flattened face of the larger one.

When microsomal particles were stained with uranyl acetate (which in many situations appears to act as a preferential stain for nucleic acids), they showed a complex internal structure, but no indication of the cleft between the sub-units in the 70 S particles. No evidence was found for the existence of a protein shell around a nucleic acid core in the particles, again in contrast to some small spherical viruses.

These results will be described in detail elsewhere (HUXLEY and ZUBAY, 1960).

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VII. MAGNESIUM AND PROTEIN BINDING STUDIES ON RIBONUCLEOPROTEIN FROM RAT SARCOMA

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DIBASIC cations, particularly magnesium, are essential to the structural integrity of ribonucleoprotein particles (RNP) of both plant (CHAO, 1957; Ts'O, BONNER and VINOGRAD, 1958) and animal (HAMILTON and PETERMANN, 1959) origin. For the study of magnesium binding, RNP from a rat tumor, the Jensen sarcoma, was purified by deoxycholate treatment and repeated washing. The 83 S form dissociated to 55 S on removal of magnesium, or associated to 122 S at high magnesium concentrations. The extent of magnesium binding (r) depended on pH and ionic strength, as well as on the concentration of free magnesium. When ultracentrifugal patterns were compared at the same r, other effects of pH and ionic strength could be evaluated. The electrophoretic mobility of 83 S was a linear function of magnesium binding. On dissociation, however, changes in mobility became quite complex; increased electrical charge was apparently offset by increased frictional drag (PETERMANN, 1960b).

Sarcoma RNP prepared by density-gradient sedimentation and washing in 0·001 M phosphate-0·0005 M $MgCl_2$ buffer, at pH 7, still carries excess protein, which can be removed by washing at pH 8 and ionic strength 0·10,

or by deoxycholate. To study protein binding, RNP purified by deoxycholate treatment was used. Its ability to bind oxyhemoglobin (HbO_2) was studied by electrophoretic and ultracentrifugal analysis and by the spectrophotometric determination of the HbO_2 left in the supernatant after sedimentation of RNP–HbO_2 mixtures. One mg of RNP bound up to 0·8 mg of HbO_2. The binding constant was reduced by increased pH or magnesium, and was extremely sensitive to ionic strength (PETERMANN, 1960a).

VIII. COMPARATIVE CYTOLOGY OF CELLS SYNTHESIZING PROTEIN

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MANY electron microscopists have shown that cells synthesizing protein usually contain a system of particle covered membranes, which has been called either endoplasmic reticulum or ergastoplasm (HAGUENAU, 1958). Cells in which these structures are seen are ones which secrete the protein, and it is often overlooked that there is another class of cells which do not contain this system of membranes, although they do synthesize protein (BIRBECK and MERCER, 1957). These are cells which retain and do not secrete the synthesized protein, and these cells contain only free RNP particles.

Amongst secretory cells themselves, there are also differences in the form in which the synthesized protein is first observed. For example, in some cells it is first seen inside the "sacs" of the endoplasmic reticulum (PALADE, 1956), on the opposite side to the RNP particles. While in other cells, particularly those that form secretory granules, the protein is first observed in the Golgi zone of the cell, remote from the endoplasmic reticulum (BIRBECK and BARRICOT, 1959). Even in those cells which retain their protein and which contain only the particles, it is impossible to observe any contiguity of particles and synthesized protein.

The interpretation of electron micrographs of cells synthesizing protein is made difficult by the presence in the cell of non-particulate RNA, which is not seen. Similarly it is possible that precursor proteins may also be invisible in the micrograph.

IX. X-RAY DIFFRACTION STUDIES ON RIBOSOMES FROM VARIOUS SOURCES

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X-RAY diffraction photographs have been taken of unoriented specimens of preparations of ribonucleoprotein particles from rat liver, yeast, and *E. coli*.

In all three cases, wet gels of these particles give clear indications of an

RNA pattern, and we conclude that the structure of the RNA in the particles is very similar to that of extracted RNA.

After drying or freeze-drying, the diffraction patterns bear no resemblance to that of dried, isolated RNA, but are protein-like in character and identical in all three cases. However, on re-wetting, the original wet gel pattern is recovered, and this effect is reversible.

In the case of the spherical viruses, Turnip Yellow Mosaic and Bushy Stunt, there are no such clear indications of an RNA pattern in wet preparations. Here it is very likely that the RNA configuration is determined by that of the protein framework.

X. ULTRASTRUCTURAL ORGANIZATION OF CYTOPLASMIC NUCLEOPROTEIN STUDIED UPON FREEZE-DRIED EXOCRINE PANCREAS CELLS

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THE 150 Å particles seen by electron microscopy in the cytoplasm of conventionally osmium fixed cells are generally accepted as the structural basis for the cytoplasmic nucleoprotein. These particles, however, are not detectable in cells fixed by freeze-drying. In the microsome fraction from the same tissue, where the particles may be identified without any fixation by means of the ultracentrifuge, they are clearly visible in the electron microscope after freeze-drying. Thus the freeze-drying does not conceal the particles, and it is established that there are no particles in the freeze-dried preparations. Bearing in mind that freeze-drying must be considered the most reliable method for preservation of the biological material, it is concluded that the particles probably do not exist in the living cells.

The particles seem to be formed rapidly by a change in the internal milieu of the cell, e.g. by homogenization or osmium fixation. The particles may thus be classified as artifacts. However, they appear in a very regular manner and they are still of great interest, as this makes it possible to identify the nucleoprotein morphologically in cells and homogenates and also to isolate this cell component (HANZON, *et al.* 1959).

XI. CHANGES IN THE FINE-STRUCTURE OF THE ENDOPLASMIC RETICULUM AND THE AMINO ACID INCORPORATION OF DIMETHYLNITROSAMINE-POISONED LIVER CELLS

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SINCE it is known that the endoplasmic reticulum (ER) is the source of the microsomes which are actively engaged in the incorporation of amino acids both *in vivo* and *in vitro*, it is of interest to look for changes, if any, in the

fine-structure of the ER in those cases in which amino acid incorporation is blocked in order to compare the functional and morphological aspects of the process of protein synthesis. A suitable system for such an investigation is provided by the liver of DMNA-treated rats in which the *in vivo* amino acid incorporation has been shown to be blocked for at least 50 per cent early after the administration of DMNA (MAGEE, 1958). After it was found (BROUWERS and EMMELOT, 1960) that the *in vitro* incorporation of [1 $-^{14}$C] leucine into the protein of the combined microsomal–supernatant fraction—prepared from livers of adult rats receiving an intravenous injection of 50 or 100 mg DMNA/kg body wt 3–20 hr previously—was markedly inhibited (50–100 per cent), an attempt was made to find which of the reactions involved in amino acid incorporation was blocked. Since the *in vitro* labelling of the soluble RNA (pH 5 enzyme), prepared from the DMNA-treated livers, by amino acid was hardly affected, it appeared likely that the incorporation block was exerted at the level of the RNP-particles. The mechanism of action by which the latter effect is brought about has been tentatively devised from indirect evidence which showed that protein synthesis in hepatoma tissue was not inhibited by adding DMNA *in vitro*, in contrast to that of liver tissue, while hepatoma microsomes, in contrast to liver microsomes, did not catalyse the N-demethylation reaction which formed formaldehyde from DMNA in the presence of the necessary co-factors. The latter enzyme is probably located in the membrane component of the microsomes, i.e. in the double membranes of the ER. Thus, the reaction of formaldehyde, or that of the intermediary and very reactive methylol derivative of DMNA, with reactive groups of the microsomes (either the RNP-particles or the membranes if the latter do play any part in protein synthesis) might be responsible for the observed inhibition.

Concomitant observations with the electron microscope showed that the ER of the liver cells became progressively (dose: 50–100 mg DMNA/kg rat—time: 3–20 hr) disorganized as a result of the *in vivo* treatment; "swelling" leading to vacuolization and detachment of RNP-particles was observed (EMMELOT and BENEDETTI, 1960). In addition to these changes, nuclei and mitochondria were not affected, but glycogen disappeared, whereas small lipoid granula and large lipoid inclusions appeared within the ER vesicles and scattered throughout the cytoplasmic matrix respectively. The microsomal pellet prepared from the homogenate of DMNA-treated livers showed corresponding alteration in fine-structure. In a number of cells (10 per cent as a preliminary approximation) of the liver, 20–24 hr after the highest DMNA dose, enormous masses of closely and parallel packed double membranes of the agranular (smooth) type ("Nebenkern") next to, or in continuity with, less elaborate (probably newly-formed ER) were observed. A hypertrophy of the smooth ER in the form of a tubular system was also observed in the latter cells. The electron microscopical observations were interpreted to mean that DMNA affected the ER, the main centre of protein synthesis, primarily. It remains to be decided whether the detachment of the RNP-particles, their chemical alteration or the changes in the membrane component are responsible for the observed inhibition of amino acid incor-

poration; it is not unlikely that more than one of these factors are involved. The shift in the metabolic behaviour of the DMNA-poisoned cells from glycogen and protein synthesis and storage towards lipid accumulation and the formation of lipoid membranes is suggestive of a dissociation of normal metabolic equilibria, perhaps as a result of a differential activation and inactivation of the particular enzymes which may either be located in the ER or regulated (both in regard to enzyme activity and substrate availability) by changes in the ER.

XII. RIBOSOMES AND PROTEIN SYNTHESIS IN *ESCHERICHIA COLI*

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THE INVOLVEMENT of ribonucleoprotein particles (ribosomes) in the process of protein synthesis has been studied *in vivo* by using exponentially growing cultures of bacteria. Radioactive tracers were often used. After disruption, the organisms were fractionated by a variety of techniques—centrifugation, electrophoresis, column chromatography (ROBERTS, 1958; BOLTON *et al.*, 1958, 1959; BOLTON, 1960; MCQUILLEN *et al.*, 1959; ROBERTS *et al.*, 1960; DUERKSON and COWIE, 1960).

(1) *E. coli* B was disrupted in a French pressure cell or by lysozyme/freezing-thawing; tris/succinic acid/$MgAc_2$ buffers were used; *in vivo* kinetic studies involved $^{35}SO_4$, $^{32}PO_4$, and ^{14}C amino acids.

(2) Ribosomes approximately 20 S, 30 S, 50 S, 70 S, 85 S and 100 S were observed and separated by centrifugation through sucrose gradients.

(3) The smaller ribosomes appear to be precursors to the larger; 70 S consist of 30 S + 50 S; S values greater than 70 result from change of shape and/or hydration or dimerisation of 70 S ribosomes.

(4) Particles 30 S and larger contain about 60 per cent RNA and 40 per cent protein. The RNA's of 30 S and 50 S differ slightly in base ratios but are quite different from soluble RNA.

(5) All the RNAase of *E. coli* is in the ribosomes but is latent until their structure is disrupted e.g. by urea or EDTA.

(6) Functional ribosomes can be made in the absence of DNA synthesis; ribosomal as well as soluble RNA can be made in the presence of chloramphenicol.

(7) The RNA and structural protein of the ribosomes are conserved during cell growth. Ribosomes are regenerated at an exponential rate in depleted cells but protein is synthesized at a rate proportional to the ribosome content at any time.

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† This report is based on work carried out at the Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D.C., by R. B. Roberts, E. T. Bolton, R. J. Britten, D. B. Cowie, B. J. McCarthy and K. McQuillen.

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(8) All material destined to become native soluble protein has a transient existence as "nascent protein" in association with ribosomes (particularly 70 S). The time constant for making protein is of the order of a few seconds at most.

(9) A small amount of β-galactosidase activity is firmly associated with ribosomes and is uniquely and specifically enhanced by addition of appropriate antiserum. The ternary complex of ribosome, enzyme and antibody sediments as a 70 S particle but can be precipitated by antiserum prepared against the anti-β-galactosidase serum. Fully induced cells contain a larger amount of such ribosome-enzyme complexes than do non-induced cells.

XIII. STUDIES ON MICROSOMAL PROTEIN FRACTIONS: THE INCORPORATION PROCESS AND THE COMPOSITION OF RIBONUCLEOPROTEINS

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TREATMENT of microsomes with the detergents Lubrol W and sodium perfluoro-octanoate (PFO) solubilizes many of those proteins which are only loosely, if at all, associated with ribonucleic acid (COHN and BUTLER, 1958). Two ribonucleoprotein residual fractions have been obtained: (a) the Lubrol pellet, (b) the PFO pellet obtained by the action of PFO on (a). Both retain almost all the RNA of the original microsome pellet. The mechanism of incorporation of amino acids into the proteins of the Lubrol pellet appears to be similar to that found for whole microsomes in that the presence of cell sap is required under some conditions. However, incorporation of amino acids into the PFO pellet is not enhanced by the addition of cell sap (COHN, 1959), and an energy generating system must be present.

The pattern of incorporation into microsomal protein fractions found in the intact animal (COHN and BUTLER, 1958) has been compared with that observed in a cell-free system. Microsomes were incubated with [^{14}C] amino acids in the presence of cell sap and the necessary co-factors. The microsome pellet was then isolated again and fractionated. The results showed that at all periods of incubation examined, the ribonucleoprotein fractions possessed a higher specific radioactivity than the protein of the whole microsomes, while the proteins of the Lubrol supernatant liquid were labelled to a slight extent only. These results are in contrast with the pattern of labelling obtained in the intact rat. Here, five minutes or more after the administration of [^{14}C] phenylalanine, proteins of the Lubrol supernatant liquid possessed a specific radioactivity higher than that of the ribonucleoprotein fractions.

In view of these differences in their behaviour, the amino acid composition of the microsomal, Lubrol and PFO pellets has been determined (BUTLER *et al.*, 1960). It was found that in all these preparations the total protein

possessed an excess of acidic amino acids, although the content of basic amino acids was greater in the Lubrol and PFO pellets than in whole microsomes. However, when the PFO pellet was extracted by 0·2 N-HCl, basic proteins similar to histones were found in the extract, the total percentage of basic amino acids being 25 per cent and the ratio of basic to acidic amino acids 1·3-1·5.

IV. AMINO ACID INCORPORATION IN MICROSOMES FRAGMENTS

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IT IS generally admitted that incorporation of amino acids into ribonucleoprotein particles corresponds to a stage in the protein synthesis.

We have isolated (ZALTA, 1958, 1959), from rat liver microsomes a system which incorporates amino acids and differs from these described in mammalian microsomes (HOAGLAND *et al.*, 1956). This system incorporates amino acids *in vitro* without the addition of any enzyme system. It constitutes an independent system for the incorporation of amino acids.

This system is prepared by the action of a detergent of the type aryl-alkyl-phenolpolyoxyethylene and aryl-alkylsulfonate on the microsomes. The fragments isolated represent about 10–20 per cent of the microsomal protein and contain 20 per cent of ribonucleic acid. In the electron microscope they resemble the Palade particles.

The ATP-pyrophosphate exchange in the presence of amino acids is suppressed, though the microsome fragments continue to incorporate amino acids.

We have shown the existence, in these fragments, of an enzyme system which brings about the exchange between the corresponding ribonucleoside tri-and diphosphates (ATP-ADP, GTP-GDP, CTP-CDP, UTP-UDP).

ATP and GTP are not indispensable for the incorporation of leucine, whereas UTP and CTP stimulate the incorporation.

This system presents an analogy with the system isolated from *Alcaligenes faecalis* by BELJANSKI (1958).

In the presence of a mixture of 18 amino acids the incorporation of leucine is very strongly stimulated by ATP, GTP, CTP, UTP together, and by ribonucleic acid extracted from microsomes.

Moreover, 80 per cent of the amino acids are not found in the N-terminal position, and the incorporation is inhibited by chloramphenicol.

These facts indicate that this incorporation may correspond to a true synthesis.

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XV. AMINO ACID INCORPORATION INTO ISOLATED RAT LIVER RIBOSOMES

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MANY cells contain ribonucleoprotein particles (or ribosomes) 100–250 Å in diameter, free or attached to endoplasmic reticulum, and containing about 50 per cent protein and 50 per cent ribonucleic acid (ROBERTS, 1958). It is generally accepted that these ribosomes contain ribonucleoprotein "templates" on which amino acids, activated by reactions occurring in the cell sap (HOAGLAND *et al.*, 1956; HOAGLAND *et al.*, 1957), are assembled into polypeptide chains (LITTLEFIELD *et al.*, 1955). Ribosomes have been isolated from rat liver in a state in which they incorporate amino acids into protein *in vitro* (KORNER, 1959). This communication reports several pieces of evidence which indicate that the particles used are indeed ribosomes and discusses some of the properties and requirements of the system.

Incorporation of amino acids is dependent on the presence of ATP but not of an ATP-generating system, and is enhanced by the addition of cell sap. Ribosomes are more stable than microsomes; they can, for instance, be stored at 0° for some time without loss of incorporating activity, and amino acid incorporation continues for some hours. Attempts have been made to demonstrate the need for the presence of amino acids in the system for incorporation to occur. The extent of incorporation of amino acids into ribosomes at different magnesium concentrations can be correlated with their sedimentation characteristics. The 78 S particles appear to be of major significance as an incorporating unit.

XVI. THE INFLUENCE OF MICROSOMAL MEMBRANES ON AMINO ACID INCORPORATION. EXPERIMENTS WITH THE MUSHROOM POISON PHALLOIDIN

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PREVIOUS experiments on liver regeneration (VON DER DECKEN and HULTIN, 1958a) raised the question whether the activity of liver microsomes in amino acid incorporation *in vitro* is directly proportional to their content of ribonucleoprotein (RNP) particles. The experiments to be described indicate that the activity of liver microsomes depends not only on the number of RNP particles but also on structural factors which may regulate the function of these particles.

Guinea pigs were treated for varied periods with sublethal to lethal doses of phalloidin (WIELAND, 1957) and the amino acid incorporation activities of different fractions of the liver were determined *in vitro* (VON DER DECKEN *et al.*, in press). (1) The activity of isolated microsomes rapidly decreased

within 2 hr of the phalloidin injection (2) RNP particles (VON DER DECKEN and HULTIN, 1958b; RENDI and HULTIN, 1960) isolated from the same microsomes did not show this decrease. Later, when macroscopic changes of the liver appeared, the activity of the isolated RNP particles was reduced. (3) In the case of the 12,000 × g supernatant fractions the picture was more complex. When the incorporation activity of such fractions from normal livers was measured at short intervals after the start of the incubation (35° C) the incorporation rate gradually increased, reaching a maximum after 6–9 min. Corresponding fractions from phalloidin-treated animals showed a maximal rate of incorporation from the very beginning of the incubation. In the first stages of phalloidin-induced inhibition, therefore, the total incorporation in a 15-min period was often higher in the phalloidin-treated preparations than in the controls. After preincubation for several minutes, however, this fraction showed an inhibition. With 12,000 × g supernatant fractions from animals which had been more severely affected by phalloidin, the incorporation was inhibited from the very beginning as in isolated microsomes.

The experiments suggest that the functions of liver microsomes in incorporation systems are influenced by structural factors which may be related to the microsomal membranes. The possibility is considered that the effects reflect a control mechanism of protein metabolism, operative also under physiological conditions.

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XVII. THE BIOSYNTHESIS OF CYTOCHROME-*C* IN ISOLATED MITOCHONDRIA

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THE DIFFICULTIES involved in interpreting the results of experiments in which cell-free systems carry out the incorporation of labelled amino acids into total protein has been described previously (BATES *et al.*, 1960). For this reason, attempts were made to develop an acellular system capable of effecting amino acid incorporation into a single protein which could be isolated and highly purified. In addition, new experimental approaches towards the mechanism of synthesis of the protein would be opened up were the amino acid sequence of this protein known (at least in part), and were the system capable of carrying out the net synthesis of the protein.

The results of experiments carried out on rat liver mitochondria showed that these subcellular particles can carry out the incorporation of labelled amino acids into total protein (MCLEAN *et al.*, 1958; REIS *et al.*, 1959) and into cytochrome-*c* (BATES *et al.*, 1960). An extension of these experiments to calf heart mitochondria, which are able to withstand incubation for lengthy periods, permitted the demonstration of the net synthesis of cytochrome-*c* (BATES and SIMPSON, 1959; *idem*, 1960). This was observed (a) by adding

labelled cytochrome-*c* to each incubation flask at the end of the reaction (isotope dilution), and (b) gravimetrically after isolation by column chromatography. The results of both procedures agree. Enzymic assay of zero-time and 12 hr samples of cytochrome-*c* gave results identical to those of an authentic sample. Cytochrome-*c* synthesis, linear with time, shows an absolute requirement for energy and for an amino acid mixture. Synthesis solely by coupling of heme and preformed protein is rendered unlikely by (a) parallel changes in valine-1-^{14}C incorporation and extent of net synthesis, and (b) agreement between the amount synthesized as calculated from valine-1-^{14}C incorporation and that derived from isotope dilution data.

To demonstrate that ^{14}C-amino acids were being incorporated into the cytochrome-*c* chain, a heme-peptide (-val-glu(NH_2)-lys-cySH-ala-glu(NH_2)-cySH-his-thr-val-glu-), located somewhere in the middle of the cytochrome chain, was isolated after peptic degradation of lysine- and valine-labelled cytochrome-*c*, and these amino acids were found in their expected positions (BATES and SIMPSON, 1959; *idem*, 1960). The results of this experiment permitted the comparison of the specific radioactivities of the two valine residues (BATES and SIMPSON, 1960), the specific activity of $valine_{10}$ was found to be 12-fold higher than that of $valine_1$. The addition to the medium of unlabelled valyl glutamic acid blocked valine-1-^{14}C incorporation into position 1 almost completely, while decreasing only slightly incorporation into position 10 (BATES *et al.*, 1960). These results taken together raise the possibility that more than one polypeptide may be involved in the synthesis of cytochrome-*c*.

Further studies indicate that a supernatant fluid obtained from sonically disrupted mitochondria by centrifugation at 100,000 × g for 8 hr is able to effect the incorporation of valine-1-^{14}C into total protein (KALF and SIMPSON, 1959). Results obtained thus far on amino acid activation in mitochondrig provide evidence for the presence of only leucine and tryptophan activatina enzymes (CRADDOCK and SIMPSON, 1960). Fractionation of mitochondrial RNA indicates the presence of at least two components, one of which has a sedimentation coefficient of about 4 S (SIMPSON and DUNN, 1960).

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(See also XXIV).

XVIII. AMINO ACID–LIPID COMPLEXES AND THEIR POSSIBLE METABOLIC ROLE

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PREVIOUS work with the hen oviduct has shown that amino acids are quickly taken up into a chloroform soluble form upon incubation of the tissue (Hendler, 1958). Kinetic experiments have shown that the amino acid in this

lipid complex is in a dynamic state (Hendler, 1959). Lipid-amino acid complexes are now known to occur in a variety of tissues (WREN and MITCHELL, 1959; HAINING, *et al.*, 1960; GABY and SILBERMAN, 1960; HUNTER *et al.*, 1959; WREN, 1959). Experimental data are given to support the following statements for such complexes obtained from hen oviduct: (1) The amino acids are not free. (2) The phenomenon is apparently shared by all amino acids. (3) The amino acid is in a metabolically dynamic state. (4) Several chromatographically distinct entities are present. (5) Some of these complexes link the amino acid by a very labile bond. (6) The complexes obtained from the lipids of the structural part of the cell are generally less polar than those from the non-particulate part. (7) Processes which inhibit protein synthesis alter the chromatographic distribution of the lipid soluble amino acids. Furthermore, it has been found that similar dynamic amino acid–lipid complexes have been obtained from the cell wall and ribosome fractions of *E. coli*. These are also contained in a mixture of chromatographically distinct complexes.

The present working hypothesis considers these complexes as possibly involved in the cell at sites where amino acid may have to come into contact with lipoprotein structures. The two most obvious possibilities are amino acid transport and protein synthesis.

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XIX. OBSERVATIONS ON THE SITE AND MECHANISM OF PROTEIN SYNTHESIS IN *B. MEGATERIUM*

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STUDIES with *B. megaterium* (BUTLER *et al.*, 1958; HUNTER *et al.*, 1959) have led to the conclusion that the primary site of protein synthesis is present in material which sediments at comparatively slow speeds with the cytoplasmic membrane, after osmotic lysis of protoplasts. This fraction contains 5–10 per cent of the RNA of the cell. It leaves in the cytoplasm very little material with $S > 14$, provided the ionic strength of the medium is maintained at physiological levels. This "membrane fraction" is clearly composite, and electron micrographs show that it consists largely of membrane fragments with a highly granular appearance which in some respects resemble mammalian microsomes. It is, therefore, reasonable to conclude that in this case the ribosomes are closely associated with the membranes and not easily detached from them. After washing the membrane fraction with dilute buffers, however, 40 S ribonucleoprotein particles can be detected in the ultra-centrifuge and prepared in the usual way.

Studies on intermediate reactions in protein synthesis (HUNTER *et al.*, 1959) have shown that in many respects the whole cytoplasmic membrane fraction from *B. megaterium* resembles the combined microsome + "soluble" fractions from rat-liver. The membrane fraction contains a range of amino

acid activation enzymes. RNA is also present and it binds amino acids in a form labile to alkali and the action of RNase. In this respect it is similar to the "soluble" RNA of rat-liver. However, rapidly metabolizing complexes of lipids and amino acids, behaving chromatographically like phospholipids, have also been detected. The labelling of these complexes is inhibited by chloramphenicol under conditions where the amino acid activation enzymes remain unaffected and where the labelling of RNA with amino acids proceeds unimpaired. Thus they are not artefacts of extraction procedures: their properties are consistent with those of a precursor of labelled protein. This possibility is discussed.

XX. MYCOBACTERIAL CELL FRACTIONS AND THEIR ROLE IN PROTEIN SYNTHESIS

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THE aim of this work was to study the structure of cell fractions obtained by mechanical rupture and differential centrifugation of the cells of *Mycobacterium frieburgensis*, and to investigate their role in protein synthesis. The electronmicrograms of the $1{,}200 \times g$ and $15{,}000 \times g$ sediments showed cell walls and fragments with uniformly distributed spherical particles of ~ 150 Å in diameter attached to them. The $105{,}000 \times g$ sediment consisted only of small spherical particles of ~ 150 Å in diameter, a small number of which could be seen even in the supernatant fraction. The $105{,}000 \times g$ pellet was pink in colour and consisted mainly of RNA and protein. All fractions contained amino acid activating enzymes, but the enzymes activating individual amino acids were far from being equally distributed among them. After incubating intact cells in a culture medium in the presence of ^{14}C-tyrosine and then isolating these cell fractions, the protein of all cell fractions was found to be labelled; the specific radioactivity of the protein of the $1{,}200 \times g$ sediment was the highest. The RNA was found to be unlabelled under these conditions. We have shown previously that ^{14}C-tyrosine incorporates *in vitro* in the presence of ATP into protein *and* RNA of the $105{,}000 \times g$ supernatant (Erdös and Ullmann, 1959). Further work is in progress to correlate the findings of *in vivo* and *in vitro* experiments and to discover the participation of different cellular sub-units in the steps of protein synthesis.

XXI. BACTERIAL RIBONUCLEOPROTEIN SYNTHESIZED IN THE PRESENCE OF CHLORAMPHENICOL

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THE SIZES of certain ribonucleoprotein (RNP) particles, or ribosomes, inside *E. coli* depend upon the environment of the cells: thus, 50 S ribosomes are broken down when cells are incubated with 0·13M phosphate buffer and they

are rapidly reformed by addition of Mg^{++} ions to the culture. We have observed Schlieren boundaries due to larger ribosomes (e.g. 70 S, 100 S) only when 0·01 M magnesium acetate has been added to the extract under examination. These large ribosomes may have independent existence inside the cell if the intracellular phosphate concentration is low and that of Mg^{++} ions is high, but the sensitivity of the ribosomes to variations in salt concentrations contrasts strongly with the readiness of the bacteria themselves to grow well when similar variations are made in their growth media. It is therefore by no means certain that the large ribosomes are essential for normal metabolism. In fact these experiments, and those with chloramphenicol-inhibited bacteria, emphasise the importance of smaller ribosomes (e.g. 30 S). Other workers have suggested that chloramphenicol blocks protein synthesis by causing accumulation of low M.W. ("transfer") RNA, but we find that the RNP which accumulates inside the cells is much larger than this: it is broken down to smaller units if certain procedures (e.g. sonic treatment) are used for disrupting the cells when the extracts are prepared. Experiments will be described to show that in the presence of chloramphenicol abnormal species of RNP particles sedimenting at 14–18 S are produced which are apparently useless for protein synthesis. When cells that have been treated with chloramphenicol are incubated for 30 minutes in a growth medium containing no antibiotic, the abnormal RNP is entirely broken down and there is a sharp increase in concentration of 30 S ribosomes; as growth proceeds their concentration falls to a normal value. The first effect of chloramphenicol observed in these studies is therefore the disappearance of 30 S ribosomes and the production of 14–18 S RNP, while recovery from the antibiotic is associated with the reverse process.

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XXII. AMINO ACID ACTIVATION AND INCORPORATION AND β-GALACTOSIDASE SYNTHESIS BY SUBCELLULAR FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF DIGITONIN-LYZED PROTOPLASTS OF *E. COLI*.

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A METHOD based on the lytic properties of digitonin on bacterial protoplasts (NISMAN and HIRSCH, 1958; NISMAN and FUKUHARA, 1959a; WACHSMAN *et al.*, 1960) has been applied for the fractionation of *E. coli* penicillin protoplasts.

Cell fractions obtained by the lysis of protoplasts of *E. coli* with digitonin (1–2 per cent) in hypertonic medium (0·5 M-sucrose, 10^{-2}M-$MgSO_4$, 10^{-2}M-Tris buffer pH 7·2) were analysed for protein, nucleic acids and amino acid activating and incorporating enzymes, as well as for specific protein synthesis (β-galactosidase).

The fraction including the cytoplasmic membrane fragments which sediments at 30,000 × g and contains an average of 50 per cent of the cell protein, 40 per cent of the cell ribonucleic acid and 80 per cent of

the cell deoxyribonucleic acid. The amino acid incorporating activity of this fraction is greater than that of any other cell fractions and the amino acids (AA) are incorporated under the conditions in which no AA dependent pyrophosphate exchange with adenosinetriphosphate, nor AA transfer to soluble RNA could be demonstrated with the majority of AA. Essentially all of the AA activating enzymes are associated with the supernatant of $30,000 \times g$ centrifugation. The latter (S_1), when submitted to a high speed centrifugation under $105,000 \times g$, separates into two subsequent fractions P_2 and S_2. P_2 sediment, contains the bulk of the cell free ribosomes and those of membrane origin. This fraction is able to activate some but not all of the AA and has a high specific activity for AA incorporation. S_2 supernatant contains the highest activity for AA activation. Ethanol precipitation at $-20°$ C of S_2 (HIRSCH *et al.*, 1960) gives rise to a sediment which after resuspension in Tris buffer pH 7.2,0.01 M can be split into two additional subfractions P_3 and S_3. P_3 is the insoluble part of S_2 and sediments after centrifugation at $30,000 \times g$. This fraction can be redissolved in the presence of 1–2 per cent deoxycholate or 1–2 per cent of perfluoro-octanoate (Fluorochemical F-126). P_3 contains 2–4 per cent of RNA and 1 per cent of DNA and represents 5–7 per cent of the total cell proteins. This fraction incorporates very actively all radioactive amino acids tested into protein. Also it promotes strongly the pyrophosphate-ATP exchange in the presence of all 20 different AA, and is able to transfer the radioactive AA to soluble RNA.

Fractions P_1, P_2 and P_3 (NISMAN and FUKUHARA, 1956; *idem*, 1960 FUKUHARA *et al.*, 1960) are able to synthesize β-galactosidase. This synthesis is stimulated by DNA isolated from the P_1 30 kg fraction of a constituent for β-galactosidase *E. coli* strain (K-12-3300, $i^-y^+z^+$), as well as suppressed by the DNA from an inducible but not induced for β-galactosidase strain (K-12-4000, $i^+y^+z^+$).

XXXIII. VIRAL REPLICATION AS A MODEL FOR THE STUDY OF PROTEIN AND NUCLEIC ACID SYNTHESIS

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THE VIRUS infected host-cell system offers many unique advantages for the study of protein biosynthesis in cell-free preparations. If viral protein synthesis can be obtained in such preparations, then net synthesis is easily demonstrated by immunological techniques, and the newly synthesized protein may be readily isolated. As viral protein may be purified, and possibly characterized, all conditions are present for the study of a particular protein molecule in a cell-free system. Such systems may also provide information concerning the mechanism of control of protein specificity. If the RNA template theory is correct, then the addition of a purified "template RNA" should direct the synthesis of the protein corresponding to the RNA. Viral nucleic acid can be highly purified, and may be equivalent to "template RNA" or may direct its synthesis. The study of proteins synthesized by

normal host cell homogenates in the presence of viral nucleic acid should therefore give direct evidence concerning template theories. For these reasons, a study was made of the intracellular events occurring when encephalomyocarditis (E.M.C.) virus infects Krebs II mouse ascites cells (SANDERS *et al.*, 1958). The following is a brief summary of the results of these investigations:

(1) New intracellular virus is just detectable about 4 hr after infection, and maximal viral replication occurs at 6–8 hr. Most of the intracellular virus can be isolated from the fraction containing mitochondria which sediments at 10,000 *g* for 10 min.

(2) As the amount of E.M.C. virus (an RNA virus) in the mitochondrial fraction increases with time, so the rate of [6−^{14}C]-orotic acid incorporation into its RNA increases, reaching 320 per cent of the uninfected controls at 6 hr.

(3) The pattern of incorporation into microsomal RNA is similar to that of mitochondria, but less pronounced as it contains less virus.

(4) The rate of incorporation of ^{14}C-valine into the proteins of all the various sub-cellular fractions is depressed by infection, but begins to rise appreciably from 6 hr onward, particularly in the mitochondrial fraction.

(5) The rate of incorporation of [6−^{14}C]-orotic acid into the RNA of the nucleus, which is the major site of RNA synthesis in this cell, is progressively inhibited from the beginning of infection, falling to only 10 per cent of controls at 6 hr. This inhibition is most pronounced in that fraction of nuclear RNA which is soluble in 0·1 *M* phosphate buffer, pH 7·1 (OSAWA *et al.*, 1958), and this RNA has the highest turnover rate within the cell.

(6) Infection causes a progressive decrease in the amount of the phosphate buffer-soluble RNA of the nucleus. This loss of RNA is compensated by a rise in mitochondrial RNA of a similar magnitude.

(7) Infection causes marked cytological changes in the nucleus which are apparent only 3 hr after infection.

From this and other data, an hypothesis concerning the mode of replication of E.M.C. virus has been put forward. According to this hypothesis viral RNA is synthesized within the nucleus, passes into the cytoplasm, and there directs the synthesis of viral protein. No evidence was obtained to indicate the direct involvement of ascites cell microsomes in viral protein synthesis.

Preliminary studies suggest that the synthesis of viral protein occurs in a cell-free system, so that the role of newly synthesized viral RNA in this process may be investigated.

XXIV. THE INCORPORATION OF RADIOACTIVE AMINO ACIDS BY ISOLATED RAT-LIVER MITOCHONDRIA

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RAT liver mitochondria incorporate radioactive amino acids into their protein if incubated aerobically in a medium containing sucrose, KCl, inorganic phosphate, AMP, DPN, Mg^{2+}, succinate (or α-oxoglutarate) and a

full complement of amino acids. Cell sap or pH 5 enzyme are not essential for the incorporation, which is slightly stimulated, rather than inhibited, by ribonuclease. It is sensitive to respiratory poisons, and failure in incorporation is coincident with failure in oxidative phosphorylation.

The distribution of radioactivity in the protein of various fractions obtained by differential centrifuging of disrupted mitochondria has been studied. Treatment with low concentrations of the non-ionic detergent Triton X 100 has been useful in obtaining controlled disruption of the mitochondria. Three fractions have been studied: (I) that sedimented by 10,000 $\times$g for 30 minutes, (II) that sedimented by 100,000 $\times$g for 60 minutes and (III) the final "soluble fraction" not sedimented by 100,000 $\times$g for 60 minutes. Fraction I contains 10 per cent of the total protein and 15–25 per cent of the total RNA. Fraction II contains 25 per cent of the protein and 50–70 per cent of the RNA and resembles a microsomal pellet in appearance. It is also rich in phospholipid and succinoxidase activity. The bulk of the protein and only 5–10 per cent of the RNA is found in the final supernatant fraction. After incubation for one hour in the presence of [^{14}C]valine the mitochondria were disrupted with detergent. The relative specific radioactivities of the proteins in the various fractions were: intact mitochondria 1·0, Fraction I:1·73; II: 2·05; III: 0·28. Thus 50 per cent of the total radioactive protein was found in Fraction II.

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XXV. FORMATION OF ALTERED PROTEIN IN BACTERIA TREATED WITH A PYRIMIDINE ANALOGUE

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ADDITION to growing cultures of *E. coli* of 5-fluoro-uracil, a pyrimidine analogue which can substitute for uracil in polynucleotides, leads to a series of modifications in protein biosynthesis:

(*a*) It alters the average amino acid composition of the protein, since the proteins accumulated in treated bacteria contain less proline, less tyrosine and more arginine than under normal conditions.

(*b*) It is accompanied by the synthesis of specific protein, the general properties of which are altered (e.g., formation of a protein cross-reacting immunologically with pure β-galactosidase and being biologically inactive, and of an alkaline phosphatase of normal specific enzyme activity but highly thermolabile).

(*c*) It stimulates the differential rate of synthesis of some inducible enzymes (possible "de-repression effect").

The effect of type (*c*) can be dissociated from the two others. The mechanism of action of the analogue has been studied in detail and experiments will be discussed which suggest that the formation of altered protein and changes in amino acid composition could lie on selective effects acting on the S-RNA during transfer of amino acids to the microsomes.

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